

**PIG-TO-PRIMATE
CARDIAC XENOTRANSPLANTATION**

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To Charlie

Declaration

This thesis has been completely composed by myself. The experimental work contained within this thesis was completed in September 1996.

The work contained within it has either been performed entirely by myself or by myself as a member of a research group. That part performed by the research group, and those areas in which I have received assistance, I should like to acknowledge below.

I have not submitted this thesis in candidature for any other degree, diploma or any other professional qualification.

Signed. Paul David Waterworth.

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Abstract

Clinical transplantation is an effective form of treatment for end stage organ disease. Transplantation is restricted by the number of available donor organs and this has led to an enthusiasm for xenotransplantation. The pig has many anatomical and physiological similarities to man and is therefore the most likely donor. When organs are transplanted between species of wide phylogenetic disparity, such as pig and human, they undergo a rapid and aggressive rejection process known as hyperacute rejection (HAR), which is due to preformed natural antibody activating the complement cascade resulting in endothelial cell damage. Complement activation may be inhibited by human decay accelerating factor (hDAF) which is a species specific complement regulator. Pigs transgenic for hDAF were created by microinjection of human DNA into fertilized pig oocytes. Ten control hearts, eight heterozygous hDAF transgenic hearts and one homozygous hDAF transgenic heart, were transplanted heterotopically into the abdomen of non-immunosuppressed cynomolgus monkeys. The heterozygous hDAF transgenic pig hearts had a median beating time of 123 hours (range 97-126 hours) and the control pigs hearts had a median beating time of 39 hours (range 0.4 -104 hours { $p < 0.0001$ }). The single homozygous hDAF transgenic pig heart had a beating time of 132 hours. The control group demonstrated a bimodal distribution of survival with five hearts having a median beating time of 0.96 hours (range 0.4-8 hours) and five hearts having a median beating time of 78 hours (range 69-104 hours). None of the transgenic grafts were hyperacutely rejected, all being lost due to acute vascular rejection (AVR). Half of the control grafts were hyperacutely rejected and half failed due to AVR. So it would appear that the hDAF transgene protects the xenograft against HAR but other factors may also be playing a part as suggested by the surprisingly long survival of half of the control grafts. The level of preformed total anti-pig antibodies (APAb) and anti-galactose alpha 1-3 galactose (AAG) IgM antibodies may also influence graft function and the presence of hDAF on the graft may confer some degree of protection against the elicited total APAb and elicited AAG IgM antibody mediated AVR. In addition, graft antigenicity, as measured by the amount of glycosylated sugar epitopes, is also likely to be important.

Fifteen heterozygous transgenic and five control hearts were then transplanted heterotopically into the abdomen of immunosuppressed cynomolgus monkeys. Of the transgenic hearts ten were immunosuppressed with a high cyclophosphamide (CyP) regimen (group 1) and five were immunosuppressed with a low CyP regimen (group 2). All control hearts were immunosuppressed with the high CyP regimen. The hDAF transgenic hearts in the high CyP group had a median beating time of 40 days (range 6-62 days) compared with 9 days (range 8-34 days) for those in the low CyP group.

This difference was statistically significant ($p < 0.05$). The control grafts had a median beating time of 55 minutes (range 2-180 minutes). The difference between the control grafts and the high CyP transgenics was statistically highly significant ($p < 0.001$). The difference between the control grafts and the low CyP transgenics was also statistically significant ($p < 0.05$). All control hearts were hyperacutely rejected. Only two hearts in the high CyP group were lost due to AVR, on days 6 and 62: all other hearts showed no evidence of rejection. All hDAF transgenic hearts in the low CyP group underwent AVR. These results confirm that HAR can be abrogated with the hDAF transgene in this pig-to-primate model and that prolonged survival can be achieved. Perioperative CyP would appear to be an essential component of this regimen, without which, graft loss due to antibody-mediated AVR is encountered.

Finally, hDAF transgenic pig hearts were transplanted both heterotopically, and orthotopically into immunosuppressed baboons. No graft underwent HAR and survival of heterotopic pig-to-baboon heart xenografts for up to 21 days was achieved. Survival of life-supporting hDAF transgenic pig hearts for up to nine days was achieved when immunosuppressed with CyA, CyP and steroids, and no other immune modulating strategies, and histological analysis of this graft showed normal myocardial architecture with no evidence of rejection.

We anticipate that with the advent of new immunosuppressive agents specifically targeting the anti-xenograft antibody response, improved survival of hDAF transgenic pig organs in primates will be achieved and that clinical xenotransplantation with hDAF transgenic pig organs will be possible.

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List of Abbreviations

APAb	Anti-pig antibody
AUC	Area under curve
AVR.....	Acute vascular rejection
AAG	Anti- galactose alpha 1-3 galactose
BSA.....	Bovine serum albumin
CFD.....	Complement fixation diluent
CPBT	Cardiopulmonary bypass time
CRP.....	Complement regulatory protein
CVF.....	Cobra venom factor
CyA	Cyclosporin A
CyP	Cyclophosphamide
DAF.....	Decay accelerating factor
DNA	Deoxyribonucleic acid
Gal α 1-3 Gal.....	Galactose alpha 1-3 galactose
GSL 1.....	Griffonia simplicifolia lectin 1
HAR	Hyperacute rejection
Hb.....	Haemoglobin
hDAF	Human decay accelerating factor
H+E.....	Haematoxylin and eosin
HPF.....	High power field
HSA.....	Human serum albumin
IT.....	Ischaemic time
IVC.....	Inferior vena cava
Mab	Monoclonal antibody
MAC	Membrane attack complex
MCP	Membrane cofactor protein
MP	Methyl prednisolone
NER.....	No evidence of rejection
OPD.....	O-phenylenediamine
PBS	Phosphate buffered saline
RCA.....	Regulator of complement activity
RNA	Ribonucleic acid
SCR1	Soluble complement receptor 1
SVC.....	Superior vena cava
VWF.....	Von Willebrand's factor
WCC.....	White cell count

Chapter One

Introduction

The need for xenotransplantation

Clinical transplantation has become a victim of its own success. It is an effective form of treatment for end stage organ disease not amenable to more conservative therapy. Recipients enjoy prolonged survival, improvement in their quality of life¹ and the cost benefit for society^{2,3} is such that an increasing number of patients are being referred for transplantation. The donor pool, however, has not expanded to keep pace with the rising recipient demand and the gap between the number of patients requiring transplantation and those receiving them has steadily increased.

What, therefore, can be done to improve the situation? The essential limiting factor is the number of cases of confirmed brain stem death. Currently, in the United Kingdom, this figure is approximately 1200-1350 cases per year. Of these, approximately 20% will have a medical contraindication to organ donation and, of the remainder, consent to organ donation will not be given in 35%. Thus, the maximum number of potential organ donors is 650-700 a year. It has been shown that even if organs were removed from all potential donors, demand would continue to outstrip supply.⁴

Living donors have been a source of kidneys for many years and currently represent around 4-5% of renal transplants in the United Kingdom. More recently, living related segmental liver and lung transplants have been performed. However, the use of living related donors conflicts with the ethical principle of 'primum non nocere'- first do no harm, as there is clearly a significant risk to such donors.

This short fall in donor organs has lead to an enthusiasm for xenotransplantation. The possibility of using animals as donors is attractive for a number of reasons.

- Ready availability of organs would even out the discrepancy between demand and supply.
- Increased availability of organs would mean that the current indications for transplantation could be reassessed.
- Problems of storage and ischaemic damage to organs could be minimized.

- Transplants could be planned more efficiently in line with routine work.
- Potential exists for donor organ and recipient pre-treatment to allow specific modulation of the immune response.

The barriers to xenotransplantation

Concordant and discordant xenotransplantation

Xenotransplantation can be divided into concordant and discordant depending on the phylogenetic gap between the donor and recipient species.⁵ Transplants between concordant species (e.g. chimpanzee-to-man) are rejected over a period of days in a manner analogous to first-set allograft rejection. Whereas grafts between discordant species (e.g. pig-to-man) are rejected in minutes to hours. The rapid rejection of discordant xenografts is known as hyperacute rejection (HAR) and is due to preformed natural antibody activating the complement cascade resulting in endothelial cell damage and lysis. Clearly, for purely immunological reasons it would be easier to use a concordant species as a donor for organ transplantation into man and yet there are many objections to the use of primates for organ donation (*vide infra*).

The pig as a potential donor

Although primates offer potential concordant combinations with man, there are a number of disadvantages in the use of primates. Chimpanzees⁶ and other apes are endangered species and apes share many of our behavioural characteristics, making their use ethically and socially less acceptable.^{7,8} Other monkeys, not as closely related to man, such as baboons, are plentiful but reach a maximum size (20-30 kg) yielding a heart which is too small to support an adult human circulation. It is possible that organs from baboons could be used for paediatric heart transplantation as has already been performed without success.⁹ However, ethical issues and low breeding capacity make their widespread use unlikely.

It is likely therefore, that any successful large scale xenotransplantation programme will use a non-primate species, such as the pig. The pig is generally accepted as the most likely candidate.¹⁰ It has many anatomical and physiological similarities to man.^{11,12} Perhaps the most important potential barrier to successful xenotransplantation is that some types of organs from disparate species may not function adequately in a new environment. This issue is particularly difficult to evaluate, especially in humans, because so few xenotransplants have survived for prolonged periods. It is likely that some physiological functions of xenogeneic organs will remain intact but others will not. While it seems reasonable to expect that appropriately sized hearts may function adequately in widely disparate species combinations it has been suggested that the situation might not be quite so simple. The porcine heart is essentially suspended in the thoracic cavity by its major blood vessels, with its anterior surface resting near to the sternum, and the majority of the posterior surface adjacent to the diaphragm. The human heart, in contrast, has a trapezoidal silhouette, with a markedly eccentric apex, which is formed by only the left ventricular mass.¹³ These facts may have implications for the positioning of the cardiac xenograft within the thoracic cavity during transplantation. They may also affect the postoperative performance of the transplanted heart, in that it would need to adapt rapidly to the influence of gravitational changes on blood pressure required by the human body. The problem of adaptation of the transplanted pig cardiac xenograft to differing venous and arterial pressures within the human body is unknown, and it is not likely to become known until clinical trials are performed.

It has been shown by our group¹⁴ that primates surviving with pig kidney transplants develop marked anaemia, raising the possibility that pig erythropoietin may not function properly in primates.

It is also reasonable to suspect that significant deficiencies may exist if metabolically more complex organs, such as the liver, are used for transplantation. Will pig proteins, enzymes and hormones carry out their tasks in humans?¹⁵

We know that human recipients of baboon livers have had lower levels of serum cholesterol (consistent with levels in baboons) and remarkably low levels of serum uric acid (since the baboon liver does not produce uric acid as the human liver does).

We already have clinical evidence that *ex vivo* perfusion of pig livers by blood from human patients in fulminant hepatic failure can lead to some improvement in cerebral activity, and therefore at least temporary support by a pig liver is likely to be beneficial.¹⁶ And so the physiological disparity between pig and human organs may indeed prove to be a significant barrier to clinical xenotransplantation.

Unlike monkeys, the pig is plentiful, there being over 50 million in the United Kingdom alone. They are relatively disease free compared to monkeys whose potential zoonoses include tuberculosis and the simian immunodeficiency virus. Pigs multiply rapidly, having litters of more than ten, have a gestation period of 113 days, and achieve reproductive maturity at six months. This would allow the rapid expansion to numbers capable of supplying the demand for organs. The pig grows rapidly to a size capable of supplying organs for even the largest human. Finally, since the pig already forms part of the human food chain ethical objections to its use to save lives is limited. Animal rights groups continue to object to the genetic manipulation of pigs specifically bred for transplantation purposes and this barrier would need to be overcome prior to the onset of clinical xenotransplantation. However, pig heart valves have been utilised for many years and porcine insulin is used in millions of diabetics worldwide and so it is unlikely that any ethical issues would be insurmountable.

Given that the immunological barriers may indeed be overcome there is still considerable concern about the spread of disease from animal organs to man i.e. xenozoonosis. A large number of pathogens have been recognised that reside primarily in animals but can infect humans. In general these are the least worrisome potential pathogens, since if the organisms are known, potential animal donors can be screened

to avoid their presence. Despite the presence of many potential pathogens among different donor species, there will almost certainly be less transmission of known infections by xenotransplantation than by allotransplantation. This is simply because there is much more time available to screen animals and to raise them in relative isolation in comparison to the short time available to screen potential cadaver human donors prior to transplantation. It is the risk from unknown potential pathogens which poses the important infectious barrier to xenotransplantation. This issue was highlighted by the discovery that porcine endogenous retroviruses (PERVs) may be able to infect human cells in culture.¹⁷ However, it still remains to be proven whether these viruses could actually infect humans and whether such infection could lead to transmission to other individuals or to disease. Thus far, no evidence has emerged that these agents can infect humans. So the debate about the infectious risks of xenotransplantation are not about the risk-benefit calculations for the patient receiving the transplant but about the risk for society as a whole. Since xenotransplantation provides no significant benefit to non-recipients of the transplant, even a small risk of a serious threat to public health becomes a reasonable concern.

Hyperacute rejection

When organs are transplanted between species of wide phylogenetic disparity, such as pig and human, they undergo a rapid and aggressive rejection process known as hyperacute rejection (HAR).¹⁸ This leads to the loss of vascular integrity and organ function within minutes to hours.

The key features of HAR are severe endothelial cell injury, oedema, interstitial haemorrhage and platelet thrombi leading to patchy infarction.¹⁹ The interstitial haemorrhage and oedema seen on microscopy represent disruption of the endothelial barrier, allowing the exudation of fluid and red blood cells.

a) The role of antibody

The circulating antispecies antibodies^{20,21,22} directed against xenogeneic tissues are independent of any sensitizing event and are termed 'preformed' or naturally occurring antibodies.²³ They are present in the blood of all immunocompetent humans. Their origin and physiological role is unclear. They are predominantly IgM²⁴ but preformed antispecies IgG is also present. These antibodies predominantly recognise galactose α 1-3 galactose (gal α 1-3 gal),²⁵ a sugar present on the surface of some pathogens and which is also synthesized by lower mammals,²⁶ but not by humans and some other primates. Binding of xenoreactive antibodies to gal α 1-3 gal on the cell surface oligosaccharides of endothelial cells lining pig blood vessels causes activation of the complement cascade and so triggers hyperacute rejection.

Evidence for the role of natural antibody in hyperacute xenograft rejection is provided by the observation that depletion of these antibodies, either by plasmapheresis²⁷ or immunoabsorption,²⁸ prolongs xenograft survival. Interestingly, new born baboon serum lacks natural anti-pig xenoantibody²⁹ and hyperacute rejection does not occur in the pig-to-newborn baboon combination.³⁰

The tissue distribution of the gal α 1-3 gal epitope has been studied using the IB4 lectin. This naturally occurring plant product binds specifically to galactose molecules in the 1-3 configuration. This technique has shown dense staining on almost all pig tissues and in particular, dense staining on pig endothelial cells.

b) The role of complement

The complement system consists of a group of 30 or more proteins accounting for 10% of the total serum protein. In general, the system functions as a cascade in a way analogous to the clotting cascade. The complement system is important in antibacterial defences, clearance of immune complexes and hypersensitivity reactions. The system consists of two activation arms (classical and alternative) and a single effector arm

(figure 1.1), the junction of which is the conversion of C3 to its active components by the enzyme C3/C5 convertase. This enzyme exists in two isoforms, one of which is generated by the interaction of antibody and antigen (the classical pathway) and one of which is generated by an antibody independent mechanism (the alternative pathway). The final result is the assembly of a complex macromolecule comprising several complement components, known as the membrane attack complex (MAC). MAC inserts itself into the cell membrane, creating a pore which allows free entry of solutes and the death of the cell.^{31,32,33}

The alternative pathway is activated by a variety of molecules and surfaces. This is controlled by molecules which exist both on the surface of mammalian cells and in the soluble phase.^{34,35} In the classical and alternative pathways C3 convertases also act to activate C5, a component of the lytic pathway, and are therefore known as C3/C5 convertases.

In addition to the direct damage by the lytic pathway, complement components such as C3a released into the soluble phase act as powerful chemoattractants for neutrophils. C3b bound to endothelial cells acts to dramatically increase the adherence of neutrophils.

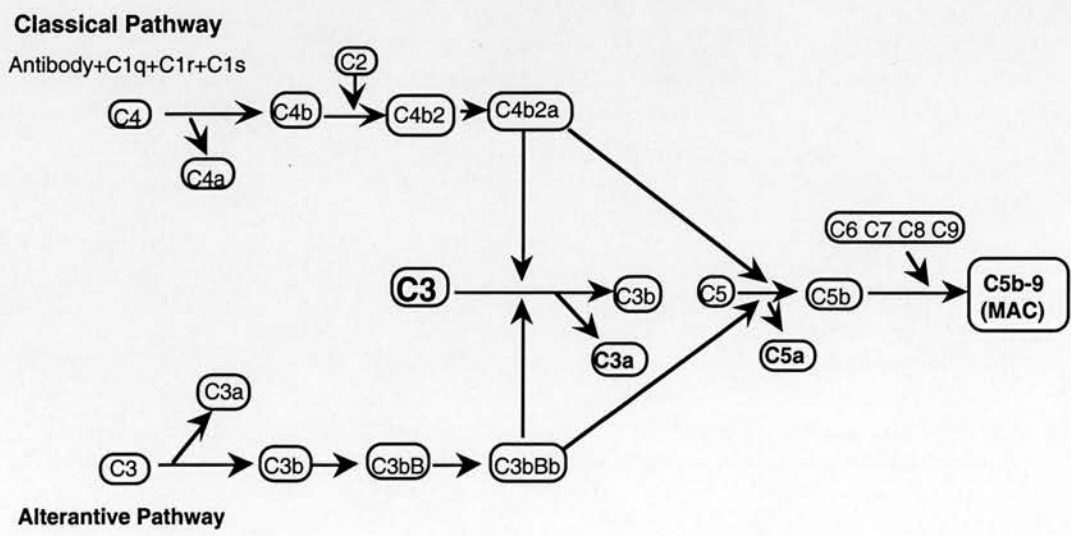


Figure 1.1. The complement cascade.

Because the complement cascade is an amplifying cascade, a series of membrane-bound and serum factors are present to protect the hosts tissues from destruction by their own complement. These factors are species specific³⁶ and known as complement regulatory proteins (CRPs) or regulators of complement activity(RCAs).³⁷ These RCAs include decay accelerating factor or CD55 (DAF),³⁸ membrane cofactor protein or CD46 (MCP)³⁹ and protectin or CD59.⁴⁰

Evidence for the role of complement in hyperacute xenograft rejection comes from the observations that:

- serum complement levels fall on revascularization of a graft that rejects hyperacutely
- complement components accumulate in rejecting xenografts⁴¹
- depletion of recipient complement, by cobra venom factor (CVF)⁴² or soluble complement receptor 1 (SCR1),⁴³ results in prolonged xenograft survival.

c) The role of endothelial cell activation

Normal "resting" endothelial cells promote a local anticoagulant environment preventing adhesion of neutrophils or platelets and the generation of thrombin. The endothelium also prevents the egress of blood constituents into the interstitium. It has been shown that the binding of xenoreactive natural antibody to gal α 1-3 gal residues on donor endothelial cell glycoproteins results in activation of the complement cascade by the classical pathway and simultaneous endothelial cell activation.⁴⁴ Once "activated" endothelial cells express a range of molecules on their cell surfaces which are capable of interacting with white cells thus bringing about the specific events of neutrophil adhesion and diapedesis.⁴⁵

Endothelial cells are important in xenotransplantation for several reasons. Firstly, since they line the blood vessels of the donor organ they are the first cell type exposed to attack by the immune system of the recipient species. The early events comprise

endothelial cell retraction, the translocation of the adhesion molecule P selectin to the cell surface, and the production of platelet activating factor. Later events such as the expression of E selectin on the cell surface require synthesis of the protein de novo and occur within hours rather than minutes.

Endothelial cell activation can promote HAR by three mechanisms.⁴⁶ Firstly, endothelial cells retract from one another leading to interstitial haemorrhage and formation of extracellular oedema. Secondly, the expression of P selectin leads to neutrophil adherence. Thirdly, activated endothelial cells become prothrombotic.

d) The role of clotting and platelets

The presence of platelet thrombi has been one of the cardinal features noted in histology of hyperacutely rejected organs. This has lead to the hypothesis that platelet thrombi associated with fibrin may be a final pathway of tissue damage in HAR.

The coagulation cascade has many analogies with the complement system. It has two potential activation arms with a common effector arm and consists of a cascade of pro-enzymes, many of them serine proteases. Furthermore, complement activation promotes the coagulation cascade. Coagulation may be initiated by the "intrinsic" pathway which is activated as a result of the interaction of factor XII with foreign surfaces. Activated factor XIIa then feeds down the pathway via factors XI, IX and X. Factor Xa is part of the common effector pathway and leads to the conversion of prothrombin to thrombin, which in turn converts fibrinogen to fibrin. The fibrin clot is stabilised by Factor XIII. Factor Xa can also be activated by the action of Factor VIIa in conjunction with tissue factor via the "extrinsic" pathway.

The formation of platelet thrombi is an equally complex process. The initial binding of platelets to the endothelium is dependent upon the interaction between platelets and Von Willebrand's Factor (VWF) on the endothelium. Following platelet activation which is promoted by thrombin, the platelets express P selectin on their surface. Interaction with fibrinogen and VWF leads to platelet aggregation and adherence to the

endothelium. There are several theoretical mechanisms by which xenografts might activate coagulation and platelet thrombi. Tissue factor is expressed abundantly on the subendothelial matrix. Thus, endothelial cell retraction, which is part of their activation, exposes tissue factor and might promote coagulation by the extrinsic pathway. Furthermore, the normal function of endothelium as an inhibitor of the coagulation cascade, may be disturbed in HAR.⁴⁷

Even if hyperacute is overcome it is likely that acute vascular rejection and chronic cellular rejection will pose significant barriers to successful clinical xenotransplantation.

Most studies in large animal species have indicated that mechanisms of cell-mediated immunity to discordant xenografts are fundamentally similar to those involved in allograft rejection but stronger. The strength of the cellular immune responses to discordant xenografts will probably be so strong that it is unlikely that it will be controlled by the types of non-specific immunosuppression used routinely to prevent allograft rejection. Indeed, it may even be necessary to induce specific immunological tolerance to some of the most antigenic xenogeneic molecules in order for xenotransplantation to become a clinical reality.

The abrogation of hyperacute rejection

The rapid destruction of discordant xenografts by HAR has made the prevention of this response a priority. Until this process has been overcome the elucidation and manipulation of further immune responses cannot be addressed. Strategies aimed at preventing HAR have taken one of two forms: either complement depletion and inhibition or antibody depletion.

Complement inhibition may be achieved with either fluid-phase complement inhibitors or with membrane-associated complement regulators of the recipient species that are expressed on the xenogeneic target cells. Examples of the former include cobra venom

factor (CVF)⁴⁸ and recombinant soluble human C receptor 1 (sCR1).⁴³ Cobra venom contains, in addition to many toxins and enzymes, a C3b-like molecule, CVF, that is resistant to human inactivating factors. This C3b analogue combines with components of the alternative pathway of complement, forming a highly stable enzyme complex that causes massive consumption of C3, Factor B, and members of the membrane attack complex, resulting in exhaustion of the complement cascade. SCR1 is a truncated recombinant form of the naturally occurring human protein CR1 (CD35, C3b/C4b receptor), which inhibits both the classical and alternative complement pathways by binding to proteins (C3b and C4b) of the multi-subunit C3 and C5 convertases and promoting the dissociation of the catalytic subunits (C2a and Bb).

Membrane associated complement regulating proteins (CRPs) such as decay accelerating factor (DAF), membrane cofactor protein (MCP) and CD59 have become the objects of great attention in the field of xenotransplantation.

Human decay accelerating factor (hDAF), also known as CD 55, is a 70 kiloDalton protein.⁴⁹ The active portion of the molecule is joined to a glycosyl phosphatidylinositol (GPI) anchor which is embedded in the cell membrane. This anchor may allow the DAF molecule to move in the plane of the membrane. In addition, because of the highly lipid soluble nature of the tail, DAF can move from the soluble phase to insert itself into the lipid membrane. DAF is present on many cells including all circulating cells and endothelial cells. A form of DAF lacking the GPI tail, exists in soluble form in plasma.

DAF works by promoting the dissociation of both the classical and alternative pathway C3/C5 convertases (figure 1.2). Thus, DAF prevents the amplification of the complement cascade associated with the binding of C3b to the human cell.

DAF from any species is an efficient inhibitor only of complement from its own species. This property is termed "species restriction" and has important implications for xenotransplantation. It has been demonstrated that human DAF is an inefficient inhibitor of pig complement.³⁶ It is also reasonable to assume that pig DAF is an

inefficient inhibitor of human complement. Thus, a pig organ transplanted into a human is vulnerable to damage by human complement as it lacks effective protection mechanisms against human complement.

Human decay accelerating factor (hDAF) has been purified and it has been demonstrated that it can be taken up into the membrane of red blood cells and then prevent the assembly of C3 and C5 convertases.⁵⁰

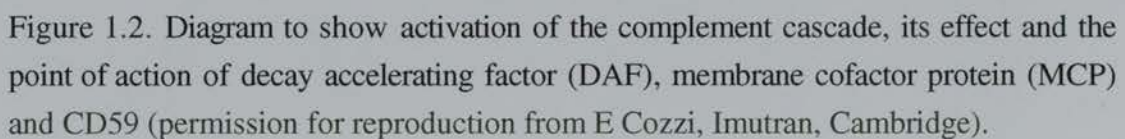
The transfer of molecules such as hDAF to cells of other species may be achieved in a number of ways. Firstly, because of its highly lipid soluble GPI tail, DAF in the serum will spontaneously insert into cell membranes.^{51,52} This process has become known as "painting". Thus, if a significant amount of hDAF can be produced in the serum, it is passively adsorbed onto cells. Secondly, the target cell can be induced to produce the foreign protein by insertion of human DNA into the cell. On a tissue culture basis this is most simply achieved by linking the human DNA to a "transfecting" virus such as an adenovirus which regularly inserts itself into the host genome.^{53,54} However, such transfection is applicable only to cells in culture. The creation of a transgenic animal requires microinjection of DNA into the cell (figure 1.3) at the single cell stage. Mice transgenic for human CD59 have been developed and shown to have biological activity, demonstrated by decreased MAC formation, when perfused ex-vivo with human blood.⁵⁵ Pigs transgenic for hDAF have been developed by our group in Cambridge^{56,57} (see later) and pigs transgenic for hCD59 have also been produced.⁵⁸ Of the many complement regulator proteins hDAF was chosen as the initial transgene for investigation as hDAF acts early in the complement cascade and therefore is likely to be the most powerful complement regulator.

Ex-vivo perfusion of hDAF transgenic pig hearts with human blood⁵⁹ and ex-vivo perfusion of hCD59 transgenic pig hearts with human blood⁶⁰ demonstrated protection of pig hearts against hyperacute rejection in both studies. Hearts from transgenic pigs, expressing hCD59 and hDAF on the cell membranes of erythrocytes, have been

transplanted heterotopically into baboons. The grafts survived for up to 30 hours compared with control pig hearts which survived for only 90 minutes.⁶¹ This strategy relied on the "painting" of vascular endothelium with hDAF as blood flows past.

In the struggle to overcome hyperacute rejection other groups have concentrated on the elimination of preformed natural antibodies against the carbohydrate epitope galactose α 1-3 galactose. This epitope is present on glycolipids and glycoproteins of all mammals up to and including New World monkeys but absent from Old World monkeys and higher primates including humans. It is the major xenoantigen which is recognised by naturally occurring antibody in human serum. The epitope is generated by galactosylation of lactobiose by α 1-3 galactosyltransferase. Blockage of this xenoantibody-xenoantigen binding can be achieved in several ways. Plasmapheresis^{62,63} or plasma exchange is an accepted form of treatment in some autoimmune conditions, and is clinically well tolerated. Several workers have shown that antibodies to xenoantigens can be reduced by plasma exchange. There is, however, a rebound of antibody within one to five days and the antibody titre can exceed the pre-depletion levels. However, in addition to removing the antibodies, plasmapheresis also removes other proteins including cytokines and complement components.

Antibody depletion can also be achieved by immunoabsorption²⁸ on antigen specific columns (Staphylococcal protein A columns which bind to the Fc portion of antibodies) and antibody blockade can be achieved by intravenous infusion of gal α 1-3 gal and gal α 1-3 gal β 1-4GlcNac.⁶⁴



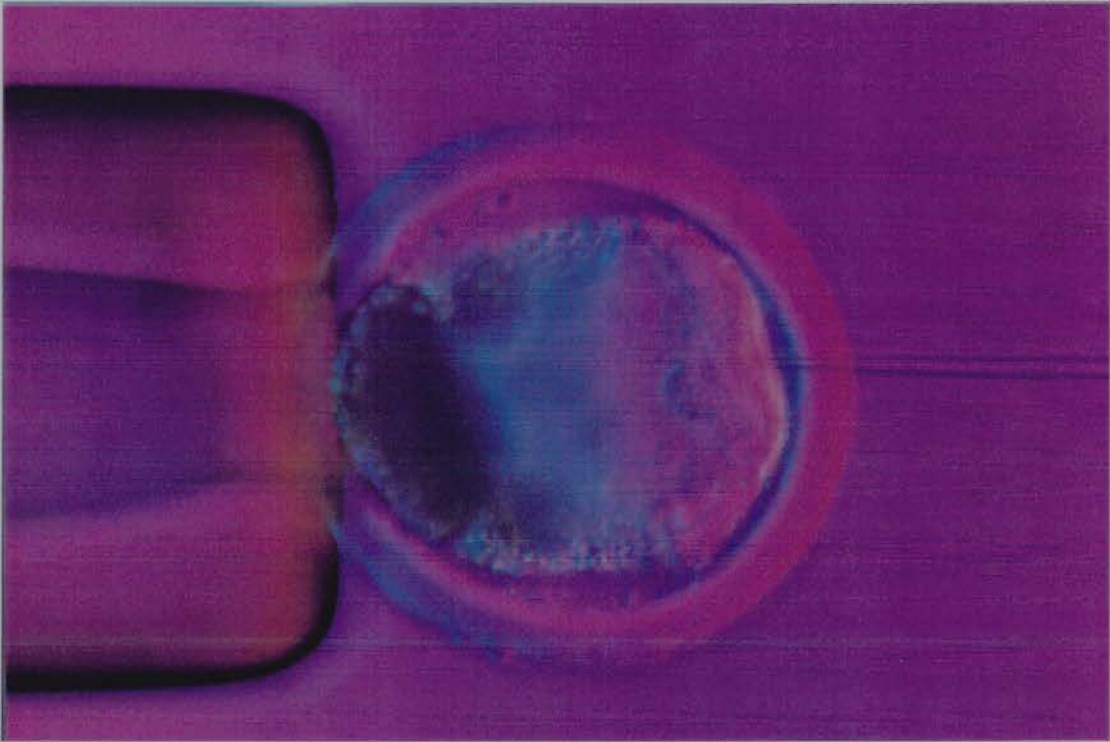


Figure 1.3. The microinjection of human DNA into a fertilized pig oocyte.

These, however, depend on partial and transient depletion of xenoantibody while donor directed genetic approaches, which potentially offer a more permanent solution, have been suggested such as knocking out the galactosyl transferase and suppression of the product of the galactosyltransferase by competition.⁶⁵ Murine monoclonal anti-idiotypic antibodies directed against human anti- α gal antibodies may also prove to be of some importance.⁶⁶

Other groups have attempted the depletion of preformed natural antibody by perfusing an organ of the donor species with the blood or plasma of the recipient. In theory, the specific anti-xenograft antibody binds to the determinants in the graft, thereby removing the specific antibodies which cause HAR. This can also be achieved by the serial transplantation of organs which also produces reduction of preformed antibodies. Unfortunately, in none of these experiments is antibody depletion complete

nor is depletion of antibody specific. The accompanying loss of other proteins, such as complement, makes the improved xenograft survival difficult to interpret.

There is also active research ongoing in the field of genetic engineering of endothelial cells to ameliorate endothelial cell activation and xenograft rejection.⁶⁷

The role of blood grouping in xenotransplantation

There is a close relationship of the pig gal α 1-3 gal to human ABO blood groups. Both are carbohydrate structures expressed on the surface of cells and there are naturally occurring antibodies to both in individuals without prior exposure to the antigen. This raises the question of blood groups in pigs⁶⁸ and their role in xenotransplantation. Only man and apes express blood groups on both red blood cells and endothelial cells.^{69,70} Lower monkeys such as baboons express ABO groups on their endothelial cells but not their red blood cells. Pigs also have blood groups, which have a limited analogy to human ABO groups, but pigs do not express these groups on their endothelium. There does not seem to be any benefit in ABO matching in xenotransplantation when pigs are used as the donor, which is not surprising since they do not express the antigen on endothelial cells.

Clinical Xenotransplantation

Year	Organ	Donor	No. of Cases	Survival
1964	Kidney	Chimpanzee	12	<9 months
1964	Kidney	Monkey	1	10 days
1964	Kidney	Baboon	1	4.5 days
1964	Kidney	Baboon	6	<2 months
1964	Heart	Chimpanzee	1	hours
1969-73	Liver	Chimpanzee	3	<14 days
1977	Heart	Baboon	1	hours
1977	Heart	Chimpanzee	1	4 days
1984	Heart	Baboon	1	4 weeks
1992	Liver	Baboon	1	70 days
1992	Heart	Pig	1	24 hours
1992	Liver	Pig	1	24 hours
1993	Liver	Baboon	1	1 month

Table 1.1. Summary of human xenograft experience.

The clinical experience to date is summarised in table 1.1. We can see from table 1.1 that the large majority of attempts at clinical xenotransplantation have involved primate kidneys. There have been only five attempts at clinical cardiac xenotransplantation, four of which have involved primates, there being only one example of non-primate to human cardiac xenotransplantation. In 1964 there were 12 chimpanzee-to-man kidney transplantations.⁷¹ The immunosuppressive regimen included, azathioprine, steroids, actinomycin-C and local graft irradiation. Prompt renal function, with diuresis was

observed in all cases. Most grafts failed within two months, but one graft survived for nine months. The grafts were lost by a process of rejection similar to that seen by human allografts. With the same immunosuppressive regimen a further six baboon-to-man kidney transplantations were performed and graft survival ranged from 19 to 60 days.⁷² Histological analysis of the grafts demonstrated a heavy infiltration of lymphocytes. The severity of rejection was greater than in the chimpanzee-to-man kidneys.

The first clinical attempts at chimpanzee-to-man liver transplantation resulted in less than 14 days graft survival.⁷³

The first attempt at clinical cardiac xenotransplantation was in 1964, when a chimpanzee heart was transplanted orthotopically into a patient with preterminal shock.⁷⁴ There is no record of any immunosuppressive therapy being used in this case. The graft functioned for only two hours. The next two cardiac xenografts were performed for the purpose of left ventricular assistance in two patients who were in cardiogenic shock following surgery for aortic stenosis. The donors were baboon and chimpanzee. The baboon heart maintained a moderate circulation for only six hours and the chimpanzee heart functioned for four days. Histological analysis of these grafts demonstrated severe rejection.⁷⁵ In 1984 a baboon heart was successful in supporting the life of a neonate for four weeks.⁹ In this case the patient was immunosuppressed with cyclosporin A and steroids and histological examination of the graft demonstrated evidence of severe rejection with a heavy cellular infiltrate of mononuclear cells.

The most recent attempt at clinical cardiac xenotransplantation was in 1992, when an unmodified pig heart was transplanted orthotopically into a patient. This graft however, failed within 24 hours, but was apparently free of hyperacute rejection at autopsy. An attempt was made to remove anti-pig antibodies from the patient by

perfusing two pig hearts ex-vivo with his blood. The patient received conventional immunosuppression with cyclosporin A, azathioprine and steroids.⁷⁶

So to date we can see that clinical xenotransplantation, although a potential solution to the donor organ shortage, has not been very successful.

The aim of this thesis is to investigate the abrogation of hyperacute rejection when hDAF transgenic pig hearts are transplanted heterotopically into non-immunosuppressed cynomolgus monkeys and to study the effect of immunosuppressive regimens on the prolongation of cardiac xenograft survival in this pig-to-primate model. In anticipation of clinical pig-to-human cardiac xenotransplantation we also aim to investigate the ability of the hDAF transgenic pig heart in supporting the life of a primate.

Chapter Two

Abrogation of hyperacute rejection in pig-to-cynomolgus monkey heterotopic cardiac xenotransplantation

Introduction

It has already been shown that hDAF protects pig hearts from hyperacute rejection when perfused with human blood *ex vivo*.⁵⁹ The aim of the experiments in this chapter was to investigate the ability of hDAF transgenic pig hearts to abrogate hyperacute rejection *in vivo* when transplanted heterotopically into a non-human primate.

The cynomolgus monkey was chosen as the non-human primate in which to test the human decay accelerating factor transgene as it has been shown *in vitro* by Tucker et al (personal communication) that cynomolgus complement activity is down regulated by human DAF by 84%, baboon complement activity by 79% and rhesus monkey complement activity by 64%. The cynomolgus monkey was thus the most appropriate of these species in which to test hDAF transgenic organs.

The United Kingdom Home Office currently restricts the number, source and species of primate which can be used for experimentation and this also influences the pig-to-primate models which can be employed. Wild-caught baboons can only be used in exceptional circumstances and captive-bred baboons are rarely commercially available. Captive-bred cynomolgus monkeys are relatively plentiful but are generally of small size, the larger animals being difficult to purchase.

Materials and Methods

All experimental procedures were performed according to the Code of Practice for Scientific Procedures in Animals from the University of Cambridge and the Guidance on the Operation of Animals (Scientific Procedures) Act 1986, Home Office, UK.

Donor selection

Heterozygous Large white/Landrace cross-piglets were selected as donors on the basis of the degree of hDAF expression and body mass. All animals were offspring of the A74 founder which had been made transgenic for hDAF by microinjection of a minigene construct (6.5kb) containing a 4kb genomic DNA fragment which incorporates the DAF promoter, the 5'-untranslated and signal peptide sequence of the DAF gene, the first exon and intron of the gene. This genomic DNA fragment was linked to the cDNA fragment that coded for the remaining exons of the DAF gene.⁵⁶

Ear clippings from each donor were used to measure tissue hDAF expression. Transgenic animals for this study were identified by DNA slot blot analysis using a random-labelled ³²P human DAF cDNA probe. Expression of the transgene was determined by Northern blot analysis, using the acid guanidium thiocyanate phenol-chloroform extraction method, and by double determinant radio-immunoassay and immunohistochemistry.⁷⁷ The expression and distribution of hDAF in the heterozygous animals shows great variability from organ to organ but hDAF expression in the hearts of A74 offspring was found to be comparable with that seen in equivalent human tissue.⁷⁸

Earlier work using autopsies from 72 pigs demonstrated that the expression of hDAF in the various tissue components of the ear clip samples correlates well with the hDAF expression in the different organs (Personal communication Chavez et al). Vascular endothelium, arterial smooth muscle, epithelium, hair follicle, eccrine glands, cartilage

and soft tissues were all stained with BRIC 216 (IBGRL Research Products, Elstree, UK), a monoclonal antibody which binds to hDAF.

Each tissue was graded from 0 to 4, according to the intensity and distribution of staining as Grade 0 = no hDAF expression, Grade 1 = equivocal staining, Grade 2 = irregular distribution, grade 3 = regular distribution, moderate intensity, and grade 4 = very strong staining for hDAF, similar to or better than human controls (figure 2.5). According to the predictive value of each parameter a score from 0 to 12 was constructed, being achieved by the sum of the values of the endothelium (maximum = 4), arterial smooth muscle (maximum = 4) and the average of the non-vascular related tissues i.e. epithelium, hair follicle, eccrine glands, cartilage and soft tissues (maximum = 4). Only "top-score" transgenic donors were used i.e. those with a score greater than 11.5 and those with a vascular endothelium score of 4. Non-transgenic Large white/Landrace cross-piglets were used as controls.

The heterotopic abdominal pig-to-cynomolgus monkey heart transplantation model⁷⁹ is a exacting model, the technical success being hindered by the paucity of space within the recipient abdomen and so wherever possible the smallest available piglets were used as donors. Practically this meant that piglets of 2-4kg were used. Donor age and sex were recorded.

Recipient selection

Healthy cynomolgus monkeys (*Macaca fascicularis*) with no evidence of intercurrent infection were selected as recipients. To maximise the technical outcome monkeys weighing greater than 2kg were selected wherever possible. Recipient age, sex and geographical origin were also recorded.

Donor operation

The donor pigs were sedated with ketamine hydrochloride (20mg/kg). Induction of anaesthesia was via inhalation of a N₂O:O₂ mixture via a face mask. The thorax and neck were prepared with antiseptic and draped. A tracheostomy was performed via a mid-line cervical incision and a suitable endotracheal tube was inserted to allow mechanical ventilation. Airway pressures and thoracic cage excursion were observed to maintain good ventilation. The endotracheal tube was manipulated if necessary and then secured in place.

Via a median sternotomy the intra-thoracic organs were exposed. The pleurae and pericardium were opened. The heart size and morphology were inspected at this stage and if function and morphology were not suitable the procedure would be abandoned. Thymectomy was performed. 2-0 vicryl (soluble braided) ties were placed around the inferior vena cava (IVC), right brachiocephalic artery and the arch of the aorta, between the right brachiocephalic and left common carotid artery. The pig was then systemically heparinised by injecting 2000 IU of sodium heparin directly into the superior vena cava (SVC). This was allowed to circulate for two minutes before proceeding. The right brachiocephalic artery was then tied and a vascular clamp was placed around the aortic arch (just distal to the origin of the right brachiocephalic artery) but left open until the IVC had been tied. The vascular clamp was then closed and a 16G cannula inserted into the ascending aorta (a silastic collar was placed 5mm from the tip of the cannula to prevent it being inserted too far and so avoiding coronary artery cannulation, tripping of the aortic valve, perforation of an aortic valve cusp or perforation of the aortic root). The heart was then vented through the SVC and IVC and 15ml/kg of cold St. Thomas's cardioplegic solution was gently administered through the aortic root until cardiac activity ceased (the heart was gently compressed to prevent distention as the cardioplegia was administered). Cold (4°C) normal saline was then placed around the heart to aid myocardial protection. The IVC was then

transected and the pig was allowed to exsanguinate into the thoracic cavity. The SVC and IVC were then ligated and the whole heart-lung block was dissected free and placed in a bowl of cold normal saline. 0-vicryl ties were placed around the left and right hilar structures (after transecting the right upper lobe bronchus which has a separate origin from the trachea) and the lungs were then amputated. The pulmonary artery was transected at its bifurcation and the aorta was transected proximal to the right brachiocephalic artery. An 0-vicryl tie was carefully secured across the roof of the left atrium allowing all bronchopulmonary tissue to be excised (care was taken not to occlude the coronary sinus or distort cardiac anatomy). The heart was protected in a fresh bowl of cold saline which was mounted on a block of ice prior to implantation. Note that the heart was kept cold at all times during the dissection.

Recipient operation

All animals were starved overnight prior to surgery. Before transfer to the operating theatre the monkey was sedated with ketamine hydrochloride (15mg/kg) given intramuscularly in combination with atropine sulphate (0.05mg/kg). Following induction of anaesthesia by intravenous injection of diazepam (1mg/kg) an endotracheal tube was inserted and the monkey was maintained on a N₂O:O₂ (1:1) mixture, with the addition of isofluorane at a concentration of 1-2% as required. A single dose of a prophylactic antibiotic was given (Synulox). An indwelling cannula was inserted into a peripheral vein for administration of fluids and drugs during the procedure. Electrocardiographic and respiratory monitoring was established.

The abdominal skin was then prepared with antiseptic solution, and sterile drapes were applied to expose the operative field. A midline laparotomy was performed, a self-retaining retractor was inserted and the bowel was carefully withdrawn from the operative field and wrapped inside warm normal saline-soaked swabs. A 2-3cm long segment of infrarenal aorta and IVC, between the inferior mesenteric vessels and the

common iliac vessels, was isolated between silastic slings, all branches being occluded with metal ligaclips. At this stage the donor heart was weighed and the vessels prepared for anastomosis. 100 iu of heparin was given intravenously and allowed to circulate for two minutes prior to occluding the IVC and abdominal aorta. The venous anastomosis (donor pulmonary artery to recipient IVC) was performed first, followed by the arterial anastomosis (donor ascending aorta to abdominal aorta), both anastomoses being performed in the same way: having occluded the vessel between the silastic slings a small longitudinal venotomy/arteriotomy was made and extended to the appropriate size to allow end to side anastomoses.⁷⁹ With the heart wrapped in a cold swab and kept cold at all times the anastomoses were performed using 7-0 prolene sutures (monofilament) and standard microvascular surgical techniques. On completion of the anastomoses the vessels were flushed with heparinised saline and all air expelled. The silastic slings around the IVC were released first followed by those around the aorta (a rapid pink flush should be seen). After checking the anastomoses for any obvious surgical bleeding, and should none be found, the slings were carefully removed (the ischaemic time noted) and the surgical field gently packed with swabs. 10-20mls of haemaccel was given intravenously (depending on the size of the animal). If the heart did not spontaneously cardiovert a 2J DC shock was occasionally required and would be repeated if necessary. If the donor heart did not return to a strong regular beat and the monkey's abdominal aortic pressure was palpably low an intra-arterial pressure recording was taken. The abdominal swabs were then removed, the surgical field was carefully examined for bleeding and thorough haemostasis was achieved. Finally, thrombin glue (Tisseal, Immuno Ltd, High Wycombe, UK) was applied around the anastomoses and the heart and abdominal contents were carefully repositioned to avoid compromising the activity of the donor heart.

Once the surgeon was satisfied that there was stable xenograft function and adequate haemostasis the wound was closed with 2-0 vicryl sutures being used to close the

rectus sheath and a 3-0 subcuticular vicryl suture to close the skin. The recipient was allowed to recover from general anaesthesia in the most appropriate position for the graft and was maintained in an incubator until sitting up, at which time it was returned to its cage.

Postoperative management

First hour

This period was critical. The animal was observed very closely during this time and kept in the operating theatre with full anaesthetic and surgical facilities available, should it be necessary to reopen the abdominal wound. Occasionally as the animal recovered and tensed its abdominal muscles the donor heart position and function would be compromised such that it became bradycardic and might stop. If such bradycardia was detected early the abdomen was reopened and the position of the donor heart was adjusted.

Hours 2-24

Postoperative analgesia (buprenorphine hydrochloride, 0.3mg/ml) was administered at the discretion of the attending veterinary surgeon. During the first 24 hours the donor heart was checked to confirm that it was beating every hour and if there was any problem with it or with the animal the surgeon was notified.

Day 2 onwards

The general physical condition and xenograft function was checked three times each day and any problem reported to the on-call surgeon.

Blood sampling and monitoring

Preoperative and postoperative total anti-pig red blood cell haemolytic antibody levels and anti-galactose α 1-3 galactose IgG and IgM levels were measured as described below.

Method for the measurement of total anti-pig red blood cell haemolytic antibodies

200 μ l clotted monkey blood was spun at 2000rpm 4°C for 10 minutes. 50 μ l of serum was then transferred to a cryovial. This was then heat inactivated along with a 50 μ l sample of human serum for 30 minutes at 56°C. Pig blood was then prepared by placing 2-3mls of fresh whole blood into a universal tube together with 7mls of complement fixation diluent ({CFD} ICN, Costa Mesa, USA). This was prepared by adding 5 tablets of oxoid CFD with 500mls of warm distilled water. The mixture was then centrifuged at 2000rpm for 6 minutes at 4°C three times. The supernatant was discarded and 1ml of red cells transferred to an eppendorf tube and spun at 13000 rpm for 2 minutes. The supernatant was again discarded and 100 μ l of red blood cells diluted with 9.9ml of CFD. Baby rabbit complement (Sera-Lab, Crawley, UK) was reconstituted by dissolving one bottle in 2mls of distilled water. This was then added to 20mls of CFD on ice. 50 μ l of test serum was then added to 50 μ l red blood cells and 50 μ l rabbit complement and there after serial dilutions of serum from 1/5-1/1280 added to the reagents. The specimens were then incubated at 37°C for one hour, spun at 1800rpm for 10 minutes and the supernatant absorbance read at 420nm in a Multiscan Plate Reader (ICN, Costa Mesa, USA). The mean absorbance was calculated for each sample and expressed as the area under curve (AUC) when compared with a standard fixed human control with an AUC equivalent to 1000.⁸⁰

Method for the measurement of anti-galactose α 1-3 galactose IgG and IgM antibodies

Preparation of affinity purified anti-galactose α 1-3 galactose IgG and IgM from pooled human serum

Quantification of anti- α gal antibody (AAG) levels in primate sera by microtitre plate methods demands a standard preparation of purified AAG of known concentration and representative binding characteristics. The antibody was prepared by fractionating human serum on a column (7x0.5cm) of bovine thyroglobulin-sepharose-4B (Pharmacia, St. Albans, UK) enriched for α - content by IB4 lectin affinity chromatography. Separation on the thyroglobulin-sepharose column was performed in the cold (4°C) at a flow rate of 1ml/minute. After applying the serum (approximately 900ml) the column was washed and specifically bound protein was batch-eluted with two consecutive high temperature incubations (in 50mM Tris-HCL, 0.15M NaCl, 0.02% NaN₃, 2x5 minutes at 57°C). The eluted material was further fractionated into IgG and IgM fractions by gel filtration (using a Waters 300SW column controlled by a Waters 650E chromatography work-station, as detailed below). The IgG and IgM was then concentrated using ultra-filtration membranes (10 kDa exclusion) from Amicon.

Separation of IgG and IgM classes in serum by Gel-filtration chromatography

Serum was spun at 10 000 rpm on a bench top centrifuge to eliminate insoluble material. 50 μ l was then fractionated by gel-filtration using a Waters 650 protein purification system linked to a Waters 717plus autosampler (fitted with a cooler unit, set at 4°C) and employing a Waters 300SW (300mm x 17mm) column. Chromatography was performed at ambient temperature in 0.1M K₂HPO₄, 0.02%

NaN₃ at a flow rate of 1ml/minute. The A280 is monitored with a Waters model 481 UV spectrophotometer and the information fed to a BBC SE 120 chart recorder.

ELISA for quantification of anti-galactose α 1-3 galactose immunoglobulin levels

Flexible 96-well microtitre plates were coated with 50 μ l (5 μ g/ml) of neoglycoconjugate galactose α 1-3 galactose β 1-4GlcNAcetyl-human serum albumin ({gal α 1-3 gal β 1-4GlcNAc-HSA} Dextra Laboratories Ltd., Reading, UK) in coating buffer (0.1 M sodium carbonate, pH 9.6) and left to absorb overnight at 4°C. The plates were then thoroughly washed with phosphate buffered saline/azide (PBS tablets from Sigma {Poole, Dorset, UK}- 1/100ml) and supplemented with 0.02% sodium azide) containing 0.1% globulin-free bovine serum albumin (BSA{Sigma, Poole, Dorset, UK}). They were then blocked with 3% globulin-free BSA in PBS/azide (2 hours at room temperature).

IgM and IgG pools were taken to 1% BSA and applied in triplicate (50 μ l per well) to the coated plate and the blocked control plate. Serial dilutions of affinity purified human anti- α -gal IgG or IgM were then applied to the same plate (2-fold from 5 μ g/ml in 1% BSA in PBS). Coated and control plates were left to adsorb antibody for 2 hours at room temperature. After this period the plates were thoroughly washed with 0.1% BSA in PBS and overlaid with 50 μ l horseradish peroxidase-conjugated anti-human IgG or anti-human IgM (Sigma, Poole, Dorset, UK), and incubated for one hour at room temperature. For development of colour, the wells were thoroughly washed with PBS and incubated with 100 μ l of the substrate *o*-Phenylenediamine (OPD {Sigma, Poole, Dorset, UK}) solution. When a strong yellow colour had formed, the plate was quenched with 50 μ l 1M H₂SO₄ and the absorbance of the wells read at 492nm with a Titertek Multiscan Plus plate reader. The mean absorbance of

each serum sample was converted to $\mu\text{g/ml}$ antibody in the serum using a standard curve constructed from the absorbances of the affinity-purified antibody preparations.

Diagnosis of xenograft rejection

Xenograft rejection was diagnosed as the cessation of cardiac xenograft pulsation determined by abdominal palpation. This diagnosis was then confirmed histologically.

Histology

Donor

Ear clippings from each donor were stained with the mouse anti-human monoclonal antibody BRIC 216 (IBGRL Research Products, Elstree, UK) to measure tissue hDAF expression (see earlier).

Recipient

At the time of explant, the transplanted grafts were stained with haematoxylin and eosin and examined histologically for each of the following:

The vascular endothelium was examined for cell retraction, white blood cell adherence, endothelial permeability and rupture.

The vascular walls were examined for inflammation and vascular lumina for congestion, thrombosis, fibrin and inflammatory cells.

Infiltration was recorded as either focal, diffuse or perivascular and the abundance of neutrophils recorded as a measure of the infiltrate aggression.

The myocardium was examined for myocyte damage, myocytolysis and focal or diffuse necrosis. Endocardial white blood cell adherence, permeability and disruption were noted as was interstitial haemorrhage and oedema.

All histological parameters were graded semi-quantitatively on a scale of 0-4, where 0 = none, 1 = equivocal, 2 = mild, 3 = moderate and 4 = severe.

Immunohistology

Cardiac tissue was snap-frozen in liquid nitrogen and stained for each of the following immunohistological parameters using mouse anti-human C3, C4, C9 and IgG monoclonal antibodies (mAbs)(Dako Ltd, High Wycombe, UK). Explanted grafts were examined for galactose α 1-3 galactose expression. This was determined by measuring the intensity of staining of biotinylated Griffonia (bandeirae) Simplicifolia Lectin 1 (GSL: Vector, California, USA) which binds specifically to galactose α 1-3 galactose epitopes. Explanted grafts were also stained for the adhesion molecule P selectin using a mouse anti-human mAb against P selectin (Immunotech, Hamburg, Germany), Von Willebrand's Factor (VWF) using mouse anti-human mAb against VWF (DAKO Ltd, High Wycombe, UK) and fibrin using mouse anti-human fibrin mAb (DAKO Ltd, High Wycombe, UK). These parameters were measured semi-quantitatively on a scale of 0-4 where 0 = no staining, 1 = equivocal staining, 2 = mild staining, 3 = moderate staining and 4 = heavy staining.

The cellular infiltrate was evaluated in each graft by counting the number of cells per five high power fields and representing this as the mean number of cells per high power field (HPF). Tissue platelets were identified using mouse anti-human mAb against P selectin (Immunotech, Hamburg, Germany). Neutrophils were identified using mouse anti-human neutrophil mAb (DAKO Ltd, High Wycombe, UK), CD-4 T helper cells using mouse anti-human CD-4 mAb (DAKO Ltd, High Wycombe, UK), CD-8 T cytotoxic cells using mouse anti-human CD-8 mAb (DAKO Ltd, High Wycombe, UK), CD-19 Bcells using mouse anti-human CD-19 mAb (DAKO Ltd, High Wycombe, UK) and CD-68 macrophages using mouse anti-human CD-68 mAb (DAKO Ltd, High Wycombe, UK).

Statistical analysis

Analysis of survival data was performed using permutation tests on the ranked data (Huntingdon Life Sciences Ltd, Huntingdon). Parametric data was analysed using the two-tailed t test assuming unequal variances (Excel version 4.0). Non-parametric data was analysed using the Mann-Whitney test (SPSS 7.5 for windows).

Results

13 control hearts, 11 heterozygous hDAF transgenic hearts and 1 homozygous hDAF transgenic heart was transplanted heterotopically into the abdomen of non-immunosuppressed cynomolgus monkeys.

The donor and recipient parameters can be seen in table 2.1. Note that there was no significant difference in the control and transgenic groups in donor organ weight, donor weight and age and recipient weight. The mean ischaemic time was similar in both groups as was the country of origin of the primates; the large majority being from the Philippines.

Parameter	Transgenics	Controls
Donor Weight (mean \pm SD)	2.5 \pm 0.6 kg	3.0 \pm 0.7 kg
Recipient Weight (mean \pm SD)	2.6 \pm 0.7 kg	3.9 \pm 1.2 kg
Donor Age (mean \pm SD)	8.7 \pm 3.8 days	8.7 \pm 3.6 days
Ischaemic Time (mean \pm SD)	1.34 \pm 0.22 hours	1.6 \pm 0.5 hours
Recipient Country of Origin	All Phillipino	Phillipino (8), Mauritian (2)

Table 2.1. Non-immunosuppressed donor/recipient parameters.

Of the "technically successful" xenografts the heterozygous hDAF transgenic pig hearts had a median beating time of 123 hours (range 97-126 hours) and the control pigs hearts had a median beating time of 39 hours (range 0.4-104 hours { $p < 0.0001$ }). The only homozygous hDAF transgenic pig heart transplanted had a beating time of 132 hours (figure 2.1), that is, greater than the best heterozygous heart. The control

group demonstrated a bimodal distribution of survival with five out of ten hearts having a median beating time of 0.96 hours (range 0.4-8 hours) and five out of ten hearts having a median beating time of 78 hours (range 69-104 hours).

The outcome can be seen in table 2.2.

Technical Outcome	Recipient	Genetics	Beating Time (hours)	Histology	Other Cause of Failure
Success					
	W57	T	126	AVR	
	V428	T	106	AVR	
	W67	T	123	AVR	
	W58	T	122	AVR	
	W65	T	125	AVR	
	W62	T	97	AVR	
	W64	T	107	AVR	
	V517	T	123	AVR	
	W567	TT	132	AVR	
	T217	C	2.18	HAR	
	T365	C	1.18	HAR	
	V427	C	76	AVR	
	V519	C	80	AVR	
	V520	C	8	HAR	
	R701	C	0.73	HAR	
	T69	C	0.4	HAR	
	R690	C	69	AVR	
	W60	C	104	AVR	
	W69	C	101	AVR	
Failure					
	R296	T	2	NER	Sacrificed
	V518	T	7	NER	Sacrificed
	V570	T	3	NER	Sacrificed
	V573	C	6.67	HAR	Sacrificed
	T405	C	4.5	HAR	Sacrificed
	V532	C	5.5	HAR	Sacrificed

Table 2.2. Outcome of non-immunosuppressed pig-to-cynomolgus monkey cardiac xenotransplantation.

T=heterozygous hDAF transgenic, TT=homozygous hDAF transgenic, C=control (non-transgenic), AVR=acute vascular rejection, HAR=hyperacute rejection, NER=no evidence of rejection.

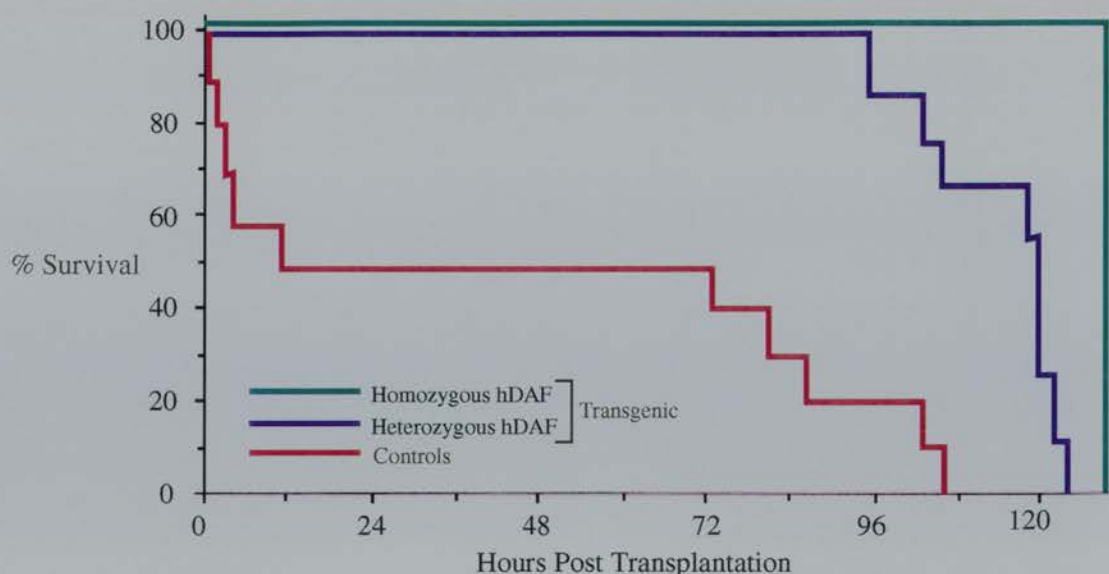


Figure 2.1. Survival of control and transgenic pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

Total anti-pig antibody levels

We can see in figure 2.2 that there was a similar pattern in both the transgenics and the long-surviving controls for both the preformed and elicited total anti-pig red blood cell haemolytic antibody (APAb) levels. In general there was a high preformed natural APAb level prior to transplantation, which fell immediately postoperatively almost to zero (presumably due to immunoabsorption onto the graft). The elicited total APAb level remained low until about the third postoperative day when it started to rise rapidly resulting in graft loss in all cases by the fifth postoperative day. We can, however, see that the control animals were less well able to tolerate high levels of elicited total APAb and indeed the mean elicited total APAb levels in the control animals at the time of sacrifice was 3260 ± 1836 (range 1788-5936) compared with a mean elicited total APAb levels in the recipients of transgenic hearts of 5629 ± 1242 (range 3957-7055). This difference did not however quite reach statistical significance. The elicited total APAb level in the only recipient of a homozygous transgenic heart at the time of sacrifice was 7185.



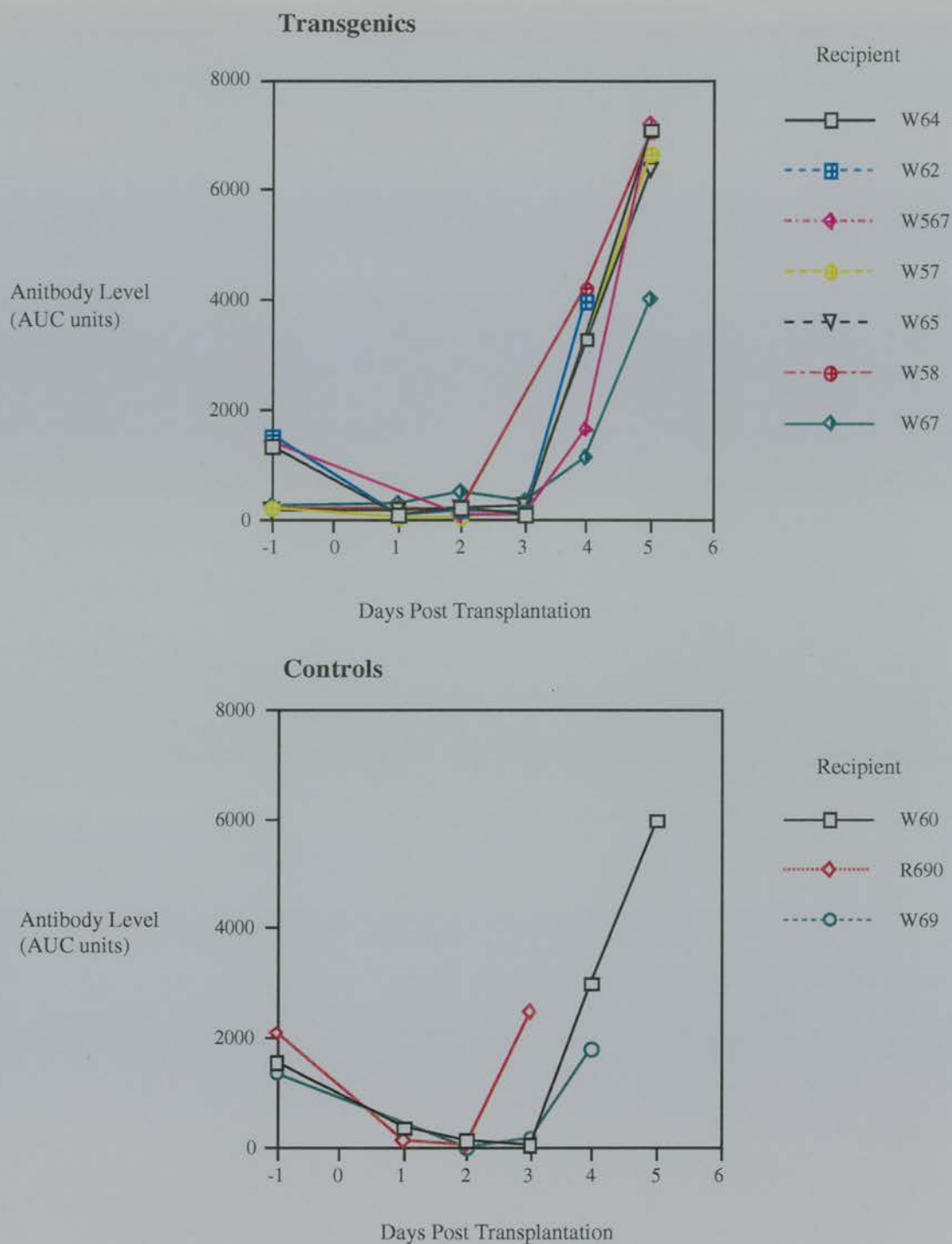


Figure 2.2. Total anti-pig red blood cell haemolytic antibody levels in non-immunosuppressed pig-to-cynomolgus monkey heterotopic cardiac transplantation.

The preformed natural APAb levels also varied in each group. In the five recipients of control hearts which stopped beating after only a few hours the mean level was 2149 (range 980-4247) compared with a mean level of 1376 (range 614-2051) for the long-surviving controls. This difference was not, however, statistically significant. The mean level for the recipients of heterozygous transgenic hearts was 518 (range 48-1508) but this data did not follow a normal distribution and therefore any statistical comparison must be made with caution. The mean preformed natural antibody level for the single recipient of a homozygous transgenic heart was 1363. Although there was a difference in the level of preformed natural total APAb for the heterozygote transgenics compared with either group of controls the difference did not reach statistical significance and there was no correlation between the preformed natural total APAb and the graft survival time for any of the groups.

Anti galactose α 1–3 galactose IgG antibody levels

We can see from figure 2.3 that the serum anti-galactose α 1-3 galactose IgG antibody levels in the cynomolgus recipients of heterotopic pig hearts remained fairly steady both before and after transplantation and the pattern was similar for both the transgenics and the long-surviving controls. All recipients tended to have levels both before and after transplantation of less than 5ug/ml. There was, however, one exception to this trend, namely recipient W62 who started with a pretransplant level of 20.4ug/ml which remained unchanged on the first post operative day, fell to a level of 13.8ug/ml by the second postoperative day and then remained steady. This suggests that we were detecting levels of preformed AAG IgG and little in the way of an elicited AAG IgG response as we might expect. There was also no significant difference in the pretransplant AAG IgG levels in those controls which underwent HAR compared with those controls which underwent AVR.

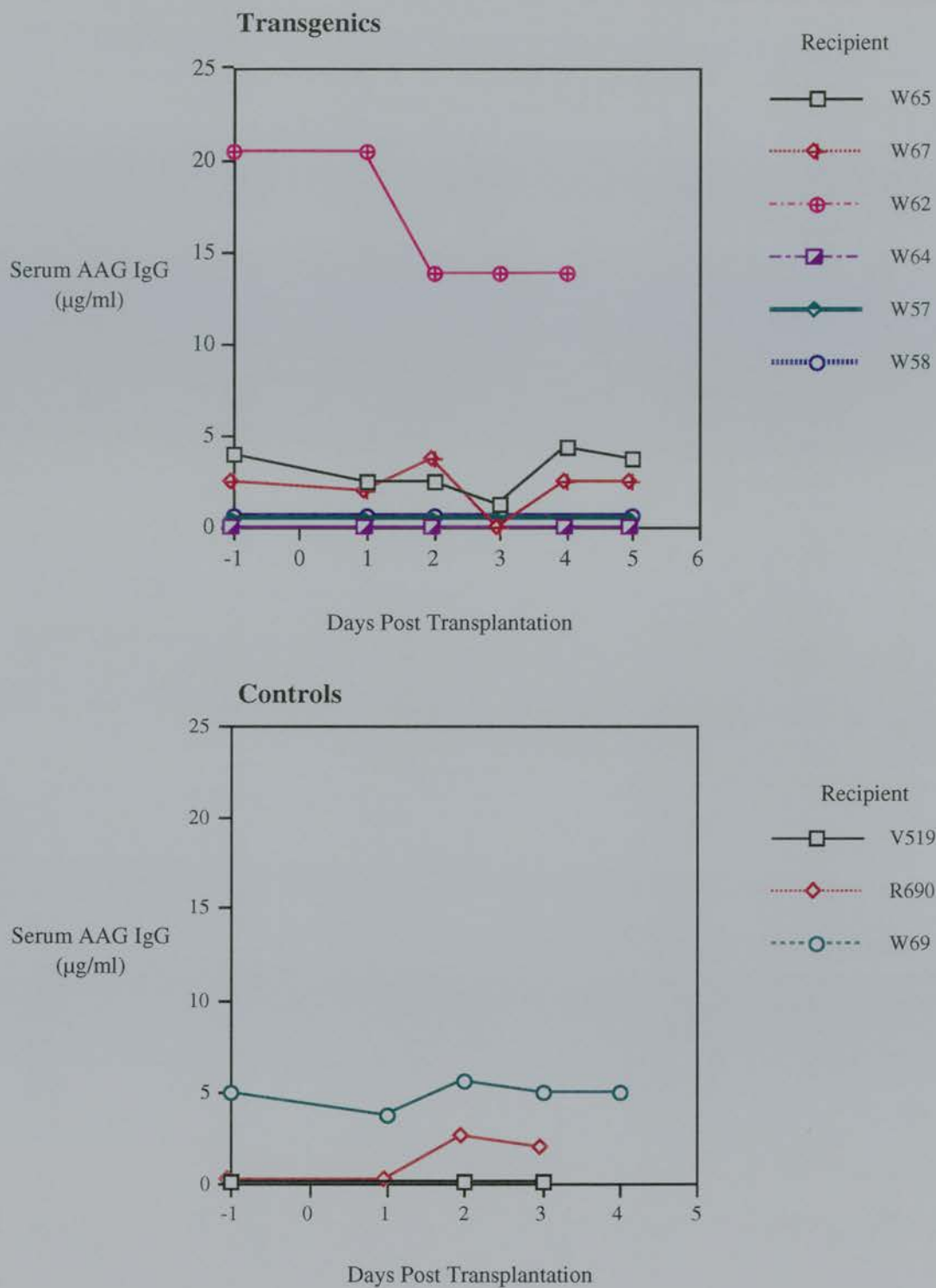


Figure 2.3. Anti-galactose α 1-3 galactose IgG levels in non-immunosuppressed pig-to-cynomolgus monkey heterotopic cardiac transplantation.

Anti-galactose α 1-3 galactose IgM antibody levels

We can see from figure 2.4 that the serum AAG IgM antibody levels in the cynomolgus recipients of heterotopic pig hearts followed a similar pattern in both the transgenics and the long-surviving controls. The mean pretransplant level of preformed AAG IgM in the transgenics was $11.0 \pm 4.1 \mu\text{g/ml}$ (range 4-17) and $24.7 \pm 14.5 \mu\text{g/ml}$ (range 8-34) in the long-surviving controls. This difference was not, however, statistically significant. There was also no significant difference in the pretransplant AAG IgM levels in those controls which underwent HAR compared with those controls which underwent AVR.

In both groups the post-transplant levels initially fell and then began to rise on the third postoperative day. In the long-surviving controls the mean AAG IgM level at the time of sacrifice was $28.7 \pm 4.2 \mu\text{g/ml}$ (range 24-32) and in the transgenic was $88.5 \pm 40.4 \mu\text{g/ml}$ (range 40-148) ($p < 0.01$).

It would appear from these results that the rise in total APAb level seen prior to rejection of the grafts was at least in part due to a rise in AAG IgM and suggests that the expression of the transgene conferred some resistance to antibody mediated acute vascular rejection.

Histology

All transgenic donor hearts scored 4 (scale of 0-4) for hDAF expression.

Histology showed florid HAR in 50% and AVR⁸¹ in 50% of the control hearts. There was no evidence of HAR in the transgenic pig hearts, all being lost due to AVR (figure 2.6).

The results of the grading for the histological parameters, immunohistological parameters and cell counts for non-immunosuppressed cynomolgus monkeys can be seen in tables 2.3 to 2.6.

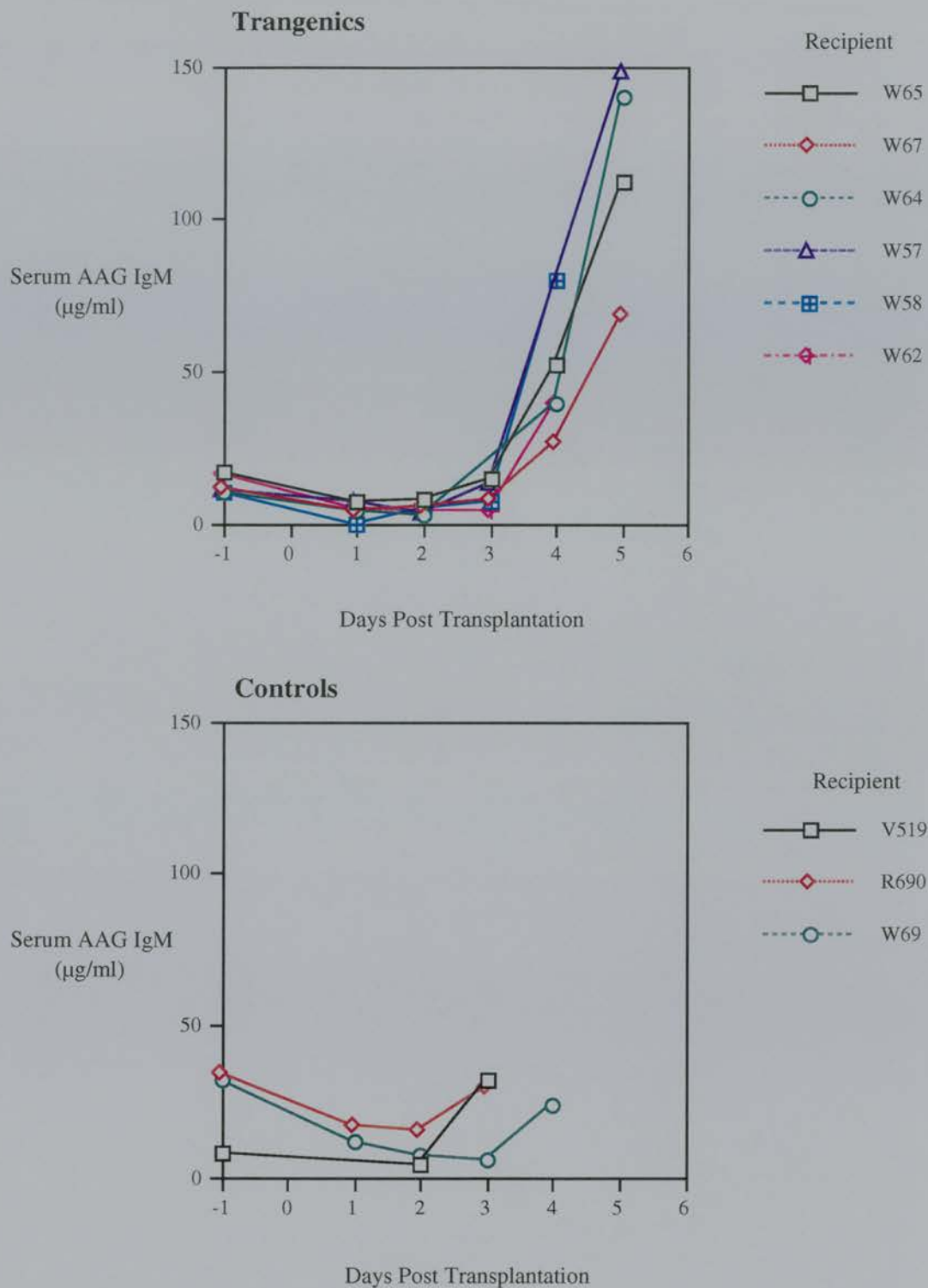


Figure 2.4. Anti-galactose α 1-3 galactose IgM levels in non-immunosuppressed pig-to-cynomolgus monkey heterotopic cardiac transplantation.

Histology of transgenic grafts compared with hyperacute rejecting control grafts

The median score for transgenic and hyperacute rejecting control grafts for endothelial white blood cell adherence, endothelial permeability, vasculitis, congestion, thrombosis, platelet thrombi, fibrin deposition, inflammatory cells, aggressive infiltrate, diffuse haemorrhage, oedema and endocardial white cell adherence was from 3-4, there being no significant difference for each of these parameters.

The cellular infiltrate was aggressive in all grafts, there being no perivascular, focal, or mild diffuse infiltrate observed. Haemorrhage was diffuse in all grafts, there being no evidence of focal haemorrhage. The median score for endocardial permeability was 2 in the transgenic group and 3 in the hyperacute rejecting control group but this difference was not statistically significant. The median score for endocardial disruption was 2 in the transgenic group and 0 in the hyperacute rejecting controls, but again this difference was not significant. Diffuse necrosis was present in all grafts, the median score for the transgenic group being 4 and that for the hyperacute rejecting control group being 2. This difference was not statistically significant. The median score for endothelial rupture was 2 in the transgenic group and 3 in the hyperacutely rejecting control group. The median score for myocytolysis was 0 in the transgenic group and 2 in the hyperacute rejecting control group. These differences did not reach statistical significance.

The only non-immunohistological parameter for which there was a statistically significant difference was myocyte damage, the median score being 0 in the transgenic group and 3 in the hyperacute rejecting control group ($p < 0.05$).

Immunohistology of transgenic grafts compared with hyperacute rejecting control grafts

All grafts had a score of 4 for endothelial C4 deposition. The median score for C3 endothelial deposition was 3 in the transgenic group and 4 in the hyperacute rejecting control group. This difference was statistically significant ($p < 0.05$). The median score for C9 endothelial deposition was 3 for the transgenic group and 4 for the hyperacute rejecting control group. This difference was statistically highly significant ($p < 0.005$). Fibrin deposition as detected immunohistologically had a median score of 4 in both groups. There was no statistical difference between the two groups for IgG deposition, staining for P selectin, deposition of VWF or GSL.1 staining.

There was no statistically significant difference in the two groups for the number of tissue platelets or tissue neutrophils expressed as the mean number/high power field.

When analyzing these results it must be remembered that we are comparing histological changes in controls which had a median beating time of 0.96 hours with transgenics which had a median beating time of 5.1 days.

Histology of transgenic and control technical failures

There were six technical failures in the non-immunosuppressed recipients: three were transgenic and three were controls. The median beating time for the transgenics was 3 hours (range 2-7 hours) and for the controls it was 5.5 hours (range 4.5-6.7 hours). This allowed us to analyse the effect of the transgene on the histological changes in control and transgenic grafts at similar time points.

All three control hearts showed clear evidence of hyperacute rejection although the actual cause of graft loss was due to technical reasons. For every histological parameter the median score was higher in the controls than the transgenics, with the exception of focal and perivascular infiltrate which was more frequently encountered in the transgenic group as an aggressive infiltrate was more frequently seen in the control

group. There was a statistically significant difference at the level of $p < 0.05$ for each of the following parameters- retracted endothelial cells, white blood cell adherence, endothelial permeability, congestion, graft thrombosis, platelet thrombi, fibrin deposition, presence of inflammatory cells, aggressive infiltrate, diffuse haemorrhage and endocardial permeability.

Immunohistology of transgenic and control technical failures

In both the transgenic group and the control group the median score for C4 deposition (figure 2.7) was 4. The median score for C3 deposition (figure 2.8) was 1 in the transgenic group and 3 in the control group. This difference was statistically significant ($p < 0.05$). The median score for C9 deposition (figure 2.9) was 0 in the transgenic group and 3 in the control group. This difference was statistically significant ($p < 0.05$). The median score for P selectin expression (figure 2.10) was 0 in the transgenic group and 2 in the control group. This difference was statistically significant ($p < 0.05$). The median score for fibrin deposition (figure 2.11) was 1 in the transgenic group and 4 in the control group. This difference was statistically significant ($p < 0.05$). There was no difference in the amount of IgG deposition (figure 2.12) or GSL.1 expression and although there was slightly more VWF expression in the controls than the transgenics (4 vs 3), this difference did not reach statistical significance.

Histology of transgenic grafts compared with long-surviving control grafts

All grafts in these groups were lost due to AVR. Despite these control animals surviving for an unexpectedly long time, the grafts were substantially damaged at the time of rejection, but for most parameters there was no statistically significant difference between the transgenic group and the long-surviving controls. Both groups

had a median score of 4 for each of the following parameters- retracted endothelial cells, endothelial white blood cell adherence, vascular congestion, intravascular thrombosis, fibrin deposition, inflammatory cells, diffuse necrosis, aggressive infiltrate and oedema. The median scores for endothelial rupture were 2 for the transgenic and 4 for the control group. The median scores for endothelial permeability were 3 for the transgenic and 4 for the control group. The median score for endocardial white blood cell adherence was 3.5 for the transgenic group and 4 for the control group and for endocardial disruption was 2 for the transgenic group and 3 for the control group. None of these differences were statistically significant. There was no myocyte damage and no myocytolysis in either group.

There was a statistically significant difference in the amount of diffuse haemorrhage ($p < 0.05$) and vasculitis ($p < 0.05$) - the scores being 3 in the transgenics and 4 in the control group. There was also a significant difference in the amount of endocardial permeability ($p < 0.05$) - the median score being 2 in the transgenic group and 4 in the control group.

There was a significant difference in the amount of platelet thrombi ($p < 0.01$) - the median score being 3 in the transgenic group and 4 in the control group.

Immunohistology of transgenic grafts compared with long-surviving control grafts

The median score for both the transgenic group and the control group was 4 for C4 deposition. The median score for the transgenic group for C3 deposition was 3 compared with a score of 4 for the control group. This difference was statistically significant ($p < 0.05$). The median score for C9 was 3 for the transgenic group and 4 for the control group. This difference was highly statistically significant ($p < 0.005$). There was a highly significant difference in the median scores for P selectin expression ($p < 0.005$) - being 3 for the transgenic group and 4 for the control group. There was

also a statistically significant difference in the amount of GSL.1 ($p < 0.01$) - the score being 3.5 for the transgenic group and 2 for the long-surviving control group. There was no difference in the median scores for fibrin and IgG deposition, that being 4 in both groups for the former and 3 in both groups for the latter. The median score for VWF deposition was 3 in the transgenic group and 4 in the control group. This difference did not reach statistical significance.

There was no difference in the number of tissue platelets per high power field. All cell counts are expressed as mean / HPF \pm SD. There were significantly more CD-4 T cells 61 ± 27 vs 17 ± 6 ($p < 0.05$), CD-8 T cells 135 ± 51 vs 53 ± 24 ($p < 0.05$) (figure 2.13) and CD-19 B cells 28 ± 12 vs 10 ± 4 ($p < 0.05$) in the long-surviving control grafts than in the transgenic grafts. There were also significantly more tissue neutrophils 196 ± 63 vs 52 ± 17 ($p < 0.01$) and CD-68 macrophages 112 ± 30 vs 50 ± 16 ($p < 0.01$) in the long-surviving control grafts than in the transgenic grafts.

Histology of hyperacute rejecting control grafts compared with long-surviving control grafts undergoing acute vascular rejection

There was significantly more endothelial rupture ($p < 0.05$) in the long-surviving controls than in the hyperacute-rejecting controls (median scores 4 vs 3). There was more myocyte damage (3 vs 0) and myocytolysis in the hyperacutely rejecting controls than in the long-surviving controls (2 vs 0) but this difference was not statistically significant. There was more endocardial disruption in the long-surviving controls than in the hyperacute rejecting controls (3 vs 0) but this difference was not statistically significant. For all other histological parameters the median scores for both groups were 3 or 4, there being no statistically significant difference between the two groups.

Immunohistology of hyperacute rejecting control grafts compared with long-surviving control grafts undergoing acute vascular rejection

The median score for GSL.1 (figure 2.14) was 3 in the hyperacutely rejecting controls and 2 in the long-surviving controls. This difference was statistically significant ($p < 0.05$). The median score for P selectin expression was 2 in the hyperacutely-rejecting controls and 4 in the long-surviving controls. This difference was not statistically significant. There was no statistically significant difference in the median scores for C3, C4, C9, IgG, VWF or fibrin deposition, the score being 3 or 4 in all cases.

The mean number of tissue neutrophils per HPF was greater in the long-surviving controls than in the hyperacutely-rejecting controls 196 ± 63 vs 88 ± 69 ($p < 0.05$). There was no significant difference in the number of tissue platelets.

Histology and Immunohistology of the homozygous hDAF transgenic pig heart compared with the heterozygous hDAF transgenic pig hearts

There was more myocyte damage (median score 4 vs 0), more myocytolysis (4 vs 0) and more diffuse haemorrhage (4 vs 3) in the homozygous heart compared with the heterozygous hearts. On the other hand, there was less endocardial adherence (2 vs 3.5), less endocardial disruption (0 vs 2) and less oedema (2 vs 4) in the homozygous heart compared with the heterozygous hearts. Finally, there was no difference in any of the other histological or immunohistological parameters.

Histological Parameter	Median Score		Statistical Significance	
	Transgenics	Controls 1	Controls 2	T vs C1
Retracted Endothelial Cells	4	4	4	NS
White Blood Cell Adherence	4	4	4	NS
Endothelial Permeability	3	4	4	NS
Endothelial Rupture	2	3	4	NS
Vasculitis	3	3	4	p <0.05
Congestion	4	4	4	NS
Thrombosis	4	3	4	NS
Platelet Thrombi	3	3	4	p <0.01
Fibrin	4	3	4	NS
Inflammatory Cells	4	4	4	NS
Myocyte Damage	0	3	0	p <0.05
Myocytolysis	0	2	0	NS
Focal Necrosis	0	0	0	NS
Diffuse Necrosis	4	2	4	NS
Perivascular Infiltrate	0	0	0	NS
Focal Infiltrate	0	0	0	NS
Diffuse Infiltrate	0	0	0	NS
Aggressive Infiltrate	4	4	4	NS
Focal Haemorrhage	0	0	0	NS
Diffuse Haemorrhage	3	3	4	p <0.05
Oedema	4	3	4	NS
Endocardial Adherence	3.5	4	4	NS
Endocardial Permeability	2	3	4	p <0.05
Endocardial Disruption	2	0	3	NS

Table 2.3. Grading and statistical significance of histological parameters for non-immunosuppressed cynomolgus monkeys. T= transgenic, C1 = Controls 1= Controls undergoing hyperacute rejection, C2 = Controls 2 = controls undergoing acute vascular rejection, NS = not significant.

Note that the transgenic grafts were substantially damaged after a median beating time of 123 hours. A similar amount of damage was also seen in the hyperacute rejecting control grafts after a median beating time of 0.96 hours. Note that when the transgenic grafts are compared with the long-surviving controls (median beating time of 78 hours) there was significantly less, platelet thrombi, vasculitis, endocardial permeability and diffuse haemorrhage in the transgenic grafts.

Immunohistological Parameters	Median Score			Statistical Significance		
	Transgenics	Controls 1	Controls 2	T vs C1	T vs C2	C1 vs C2
C3	3	4	4	p <0.05	p <0.05	NS
C4	4	4	4	NS	NS	NS
C9	3	4	4	p <0.005	p <0.005	NS
IgG	3	4	3	NS	NS	NS
P selectin	3	2	4	NS	p <0.005	NS
Von Willebrand's Factor	3	4	4	NS	NS	NS
Fibrin	4	4	4	NS	NS	NS
GSL.1	3.5	3	2	NS	p <0.01	p <0.05
Mean Number/HPF						
Tissue Platelets	11	16	38	NS	NS	NS
Tissue Neutrophils	52	88	196	NS	p <0.01	p <0.05
CD-4 T Cells	17	-	61	-	p <0.05	-
CD-8 T Cells	53	-	134	-	p <0.05	-
CD-19 B Cells	10	-	28	-	p <0.05	-
CD-68 Macrophages	50	-	112	-	p <0.01	-

Table 2.4. Grading and statistical significance of immunohistological parameters for non-immunosuppressed cynomolgus monkeys. T= transgenic, C1 = Controls 1= Controls undergoing hyperacute rejection, C2 = Controls 2 = controls undergoing acute vascular rejection, NS = not significant, HPF = high power field.

Note there was no difference in the amount of C4 deposition on transgenic and control grafts, but there was significantly more C3 and C9 deposition on control grafts. This is the pattern of complement deposition expected given the point of action of decay accelerating factor in the complement cascade. Note also significantly more P selectin staining in controls and significantly less staining for galactosylated sugars (GSL.1) in the long-surviving controls compared with the hyperacute rejecting controls.

Histological Parameter	Median Score		Statistical Significance
	Transgenics	Controls	Transgenics vs. Controls
Retracted Endothelial Cells	1	4	p <0.05
White Blood Cell Adherence	1	4	p <0.05
Endothelial Permeability	0	4	p <0.05
Endothelial Rupture	0	3	NS
Vasculitis	0	3	NS
Congestion	2	3	p<0.05
Thrombosis	0	3	p <0.05
Platelet Thrombi	0	3	p <0.05
Fibrin	0	3	p <0.05
Inflammatory Cells	1	4	p <0.05
Myocyte Damage	0	0	NS
Myocytolysis	0	0	NS
Focal Necrosis	0	0	NS
Diffuse Necrosis	0	3	NS
Perivascular Infiltrate	1	0	NS
Focal Infiltrate	1	0	NS
Diffuse Infiltrate	0	0	NS
Aggressive Infiltrate	0	3	p <0.05
Focal Haemorrhage	0	0	NS
Diffuse Haemorrhage	0	3	p <0.05
Oedema	0	4	p<0.05
Endocardial Adherence	0	3	p<0.05
Endocardial Permeability	0	2	p <0.05
Endocardial Disruption	0	2	NS

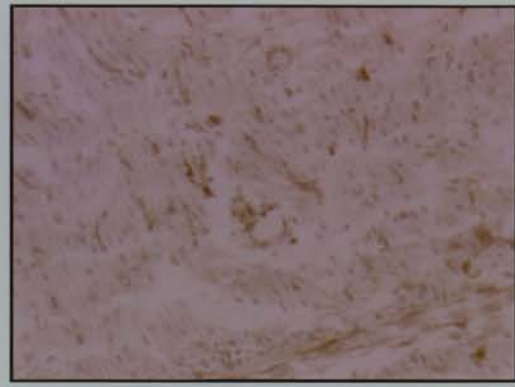
Table 2.5. Grading and statistical significance of histological parameters for non-immunosuppressed cynomolgus monkey technical failures. NS = not significant.

Immunohistological Parameter	Median Score		Statistical Significance
	Transgenics	Controls	Transgenics vs. Controls
C3	1	3	p <0.05
C4	4	4	NS
C9	0	3	p<0.05
IgG	3	3	NS
P. selectin	0	2	p<0.05
Von Willebrand's Factor	3	4	NS
Fibrin	1	4	p<0.05
GSL.1	3	3	NS

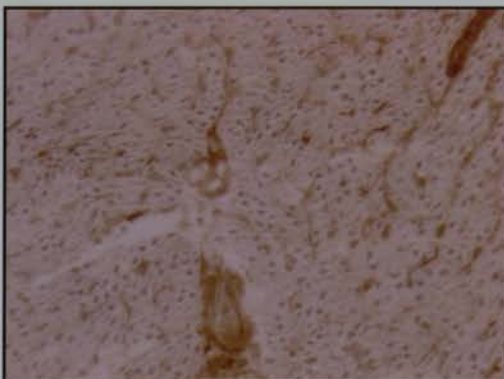
Table 2.6. Grading and statistical significance of immunohistological parameters for non-immunosuppressed cynomolgus monkey technical failures. NS = not significant.



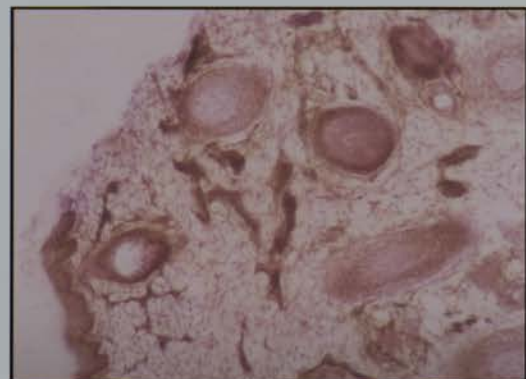
Mild hDAF expression in transgenic pig heart (x10).



Moderate hDAF expression in transgenic pig heart (x10).

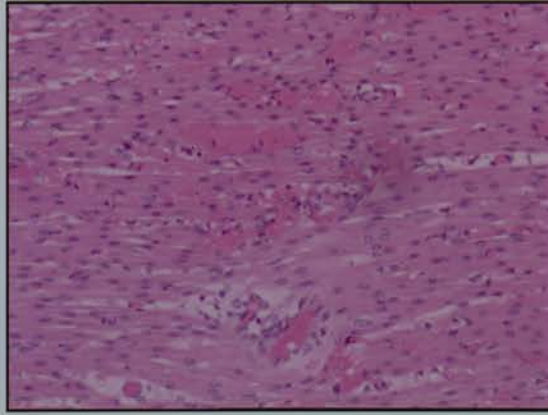


Strong hDAF expression in transgenic pig heart (x10).

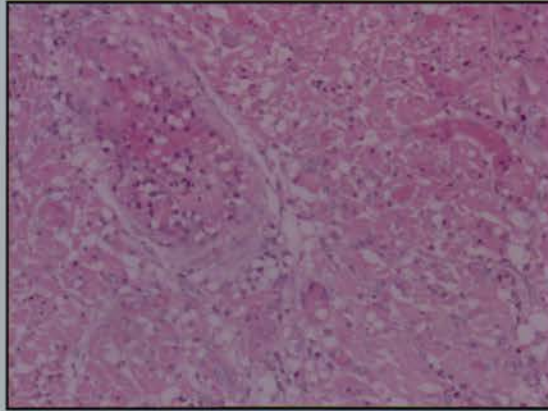


Positive control showing strong hDAF expression in human skin (x10).

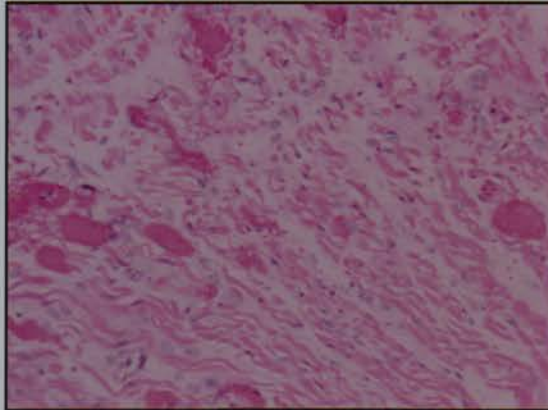
Figure 2.5. Histology photographs demonstrating grading of human DAF expression in tissues stained with BRIC 216.



Control heart (8 hours) undergoing hyperacute rejection with typical features of thrombosis, haemorrhage, oedema, muscle damage and neutrophil infiltration (x10).

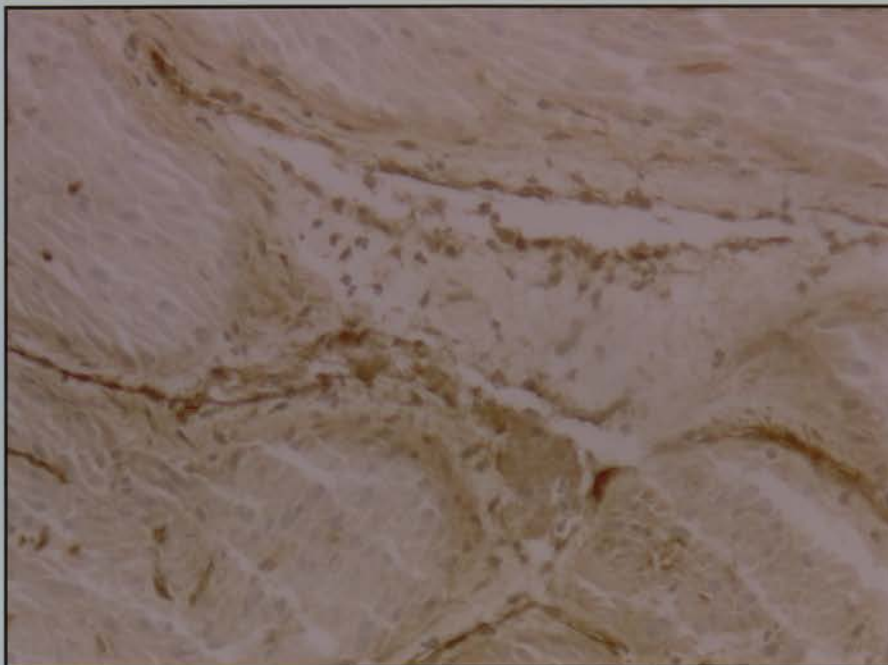


Control heart (76 hours) undergoing acute vascular rejection with typical features of thrombosis, haemorrhage, severe muscle damage, neutrophil and monocyte infiltration (x10).

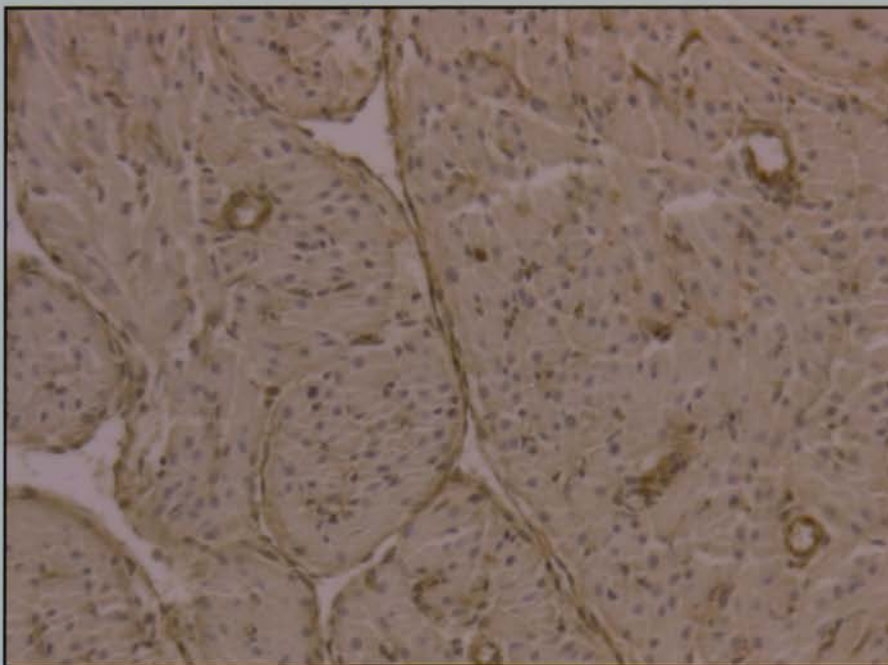


hDAF transgenic heart (123 hours) undergoing acute vascular rejection with features of thrombosis, oedema, some muscle damage, neutrophil and monocyte infiltration (x10).

Figure 2.6. Sections of cardiac tissue stained with haematoxylin and eosin from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

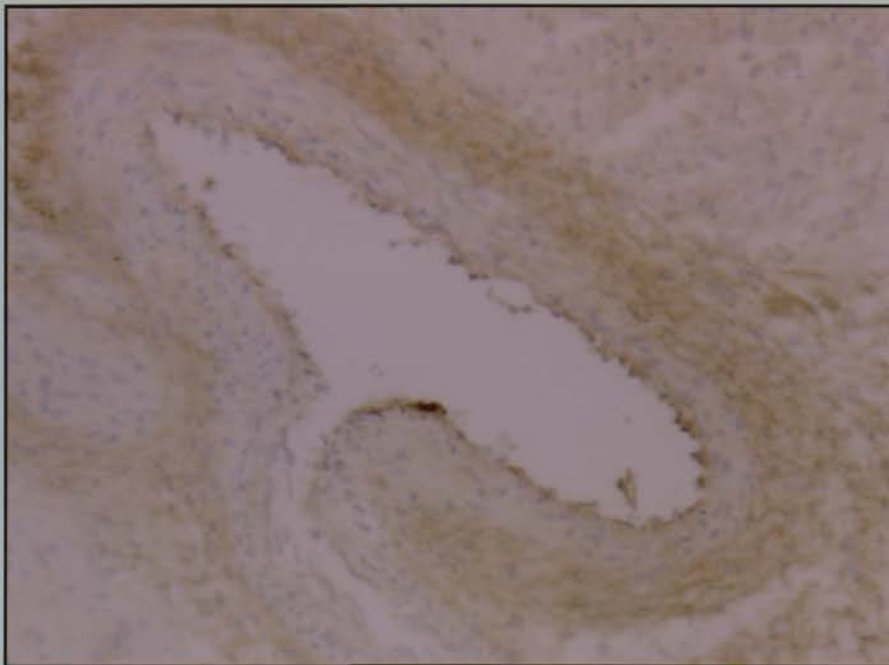


Control technical failure (6 hours) demonstrating heavy C4 deposition on the endothelium (x10).

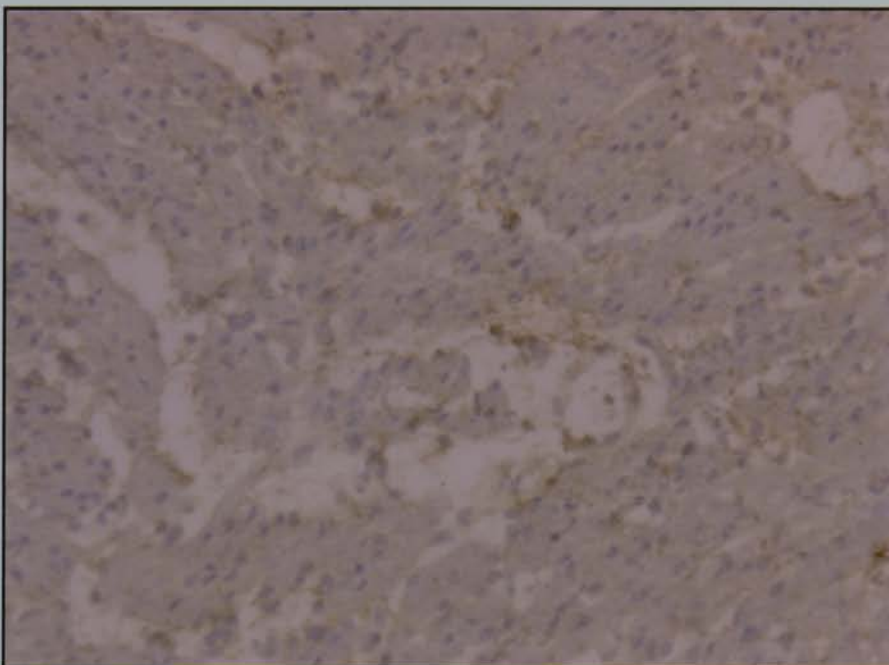


hDAF transgenic technical failure (7 hours) demonstrating heavy C4 deposition on the endothelium (x10).

Figure 2.7. Sections of cardiac tissue stained for complement component C4 from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

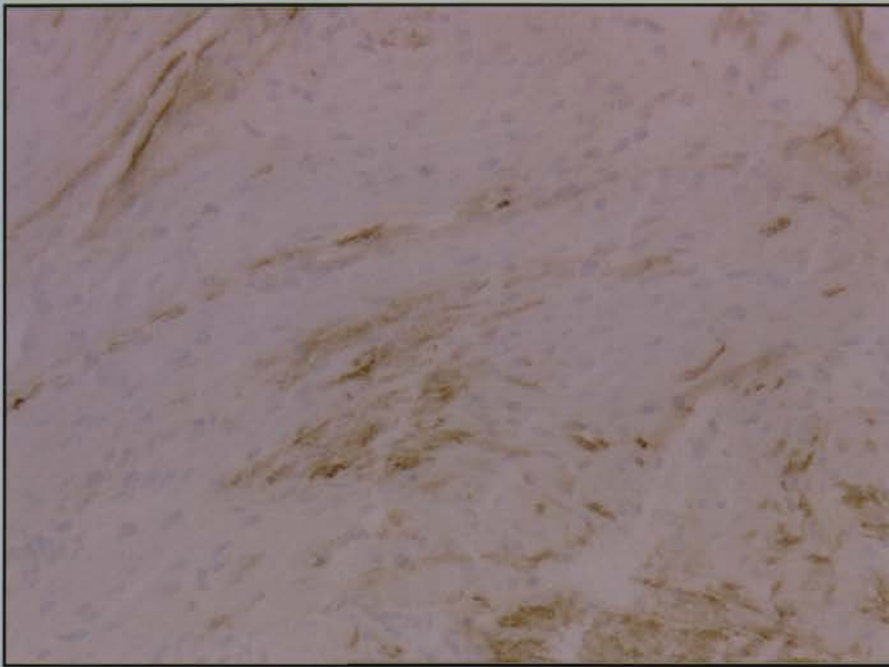


Control technical failure (6 hours) demonstrating clear C3 deposition on vascular endothelium (x20).

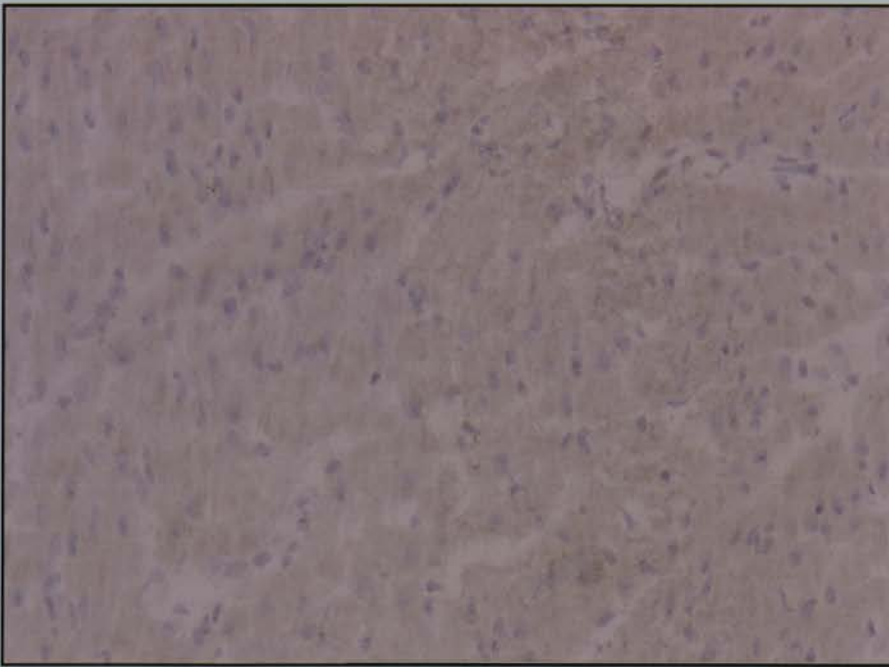


hDAF transgenic technical failure (7 hours) demonstrating mild C3 deposition on vascular endothelium (x10).

Figure 2.8. Sections of cardiac tissue stained for complement component C3 from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

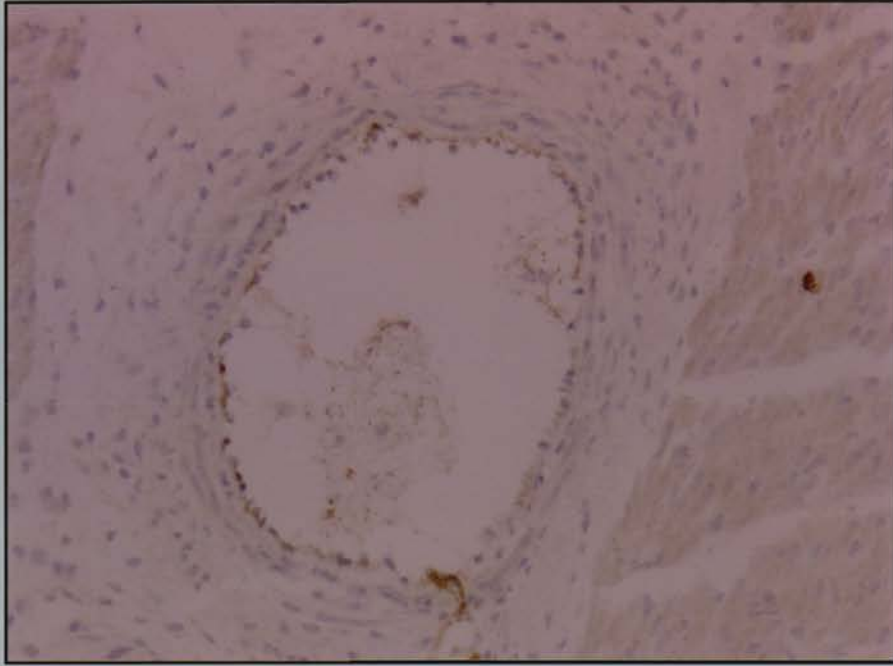


Control technical failure (6 hours) demonstrating heavy C9 deposition within vessels and necrotic tissue (x10).

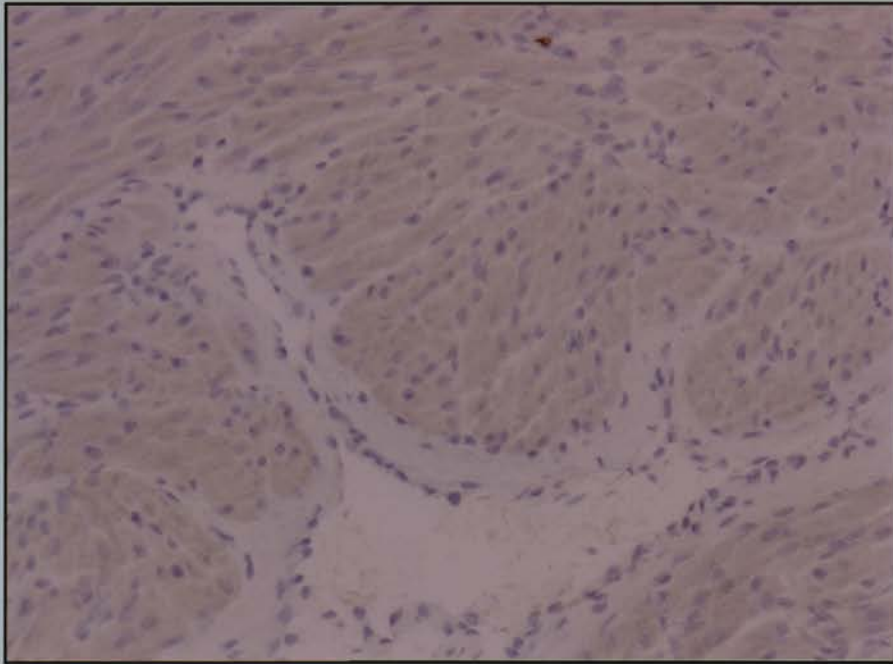


hDAF transgenic technical failure (7 hours) demonstrating no C9 deposition (x10).

Figure 2.9. Sections of cardiac tissue stained for complement component C9 from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

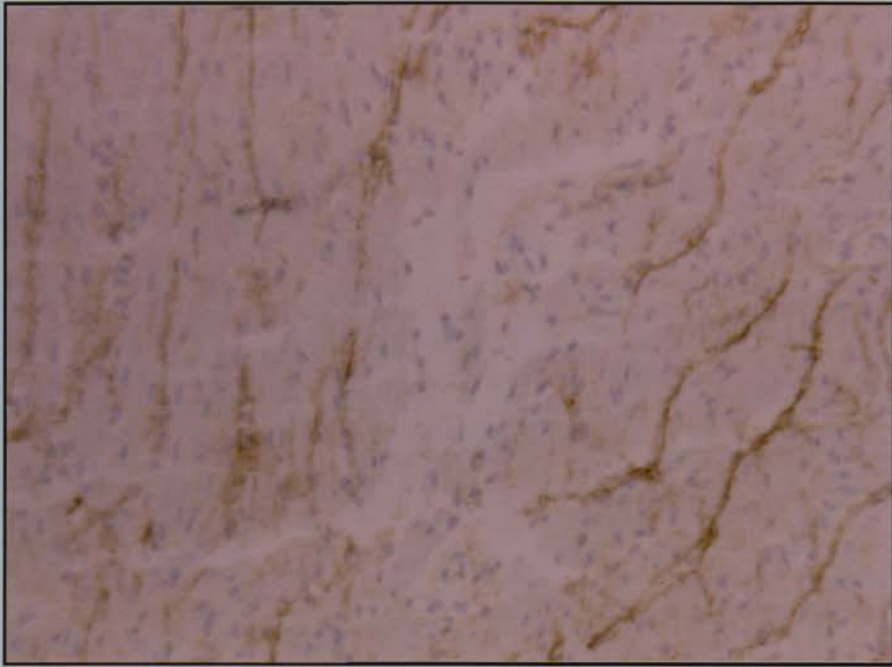


Control technical failure (6 hours) demonstrating heavy P selectin deposition within the vessel (x20).

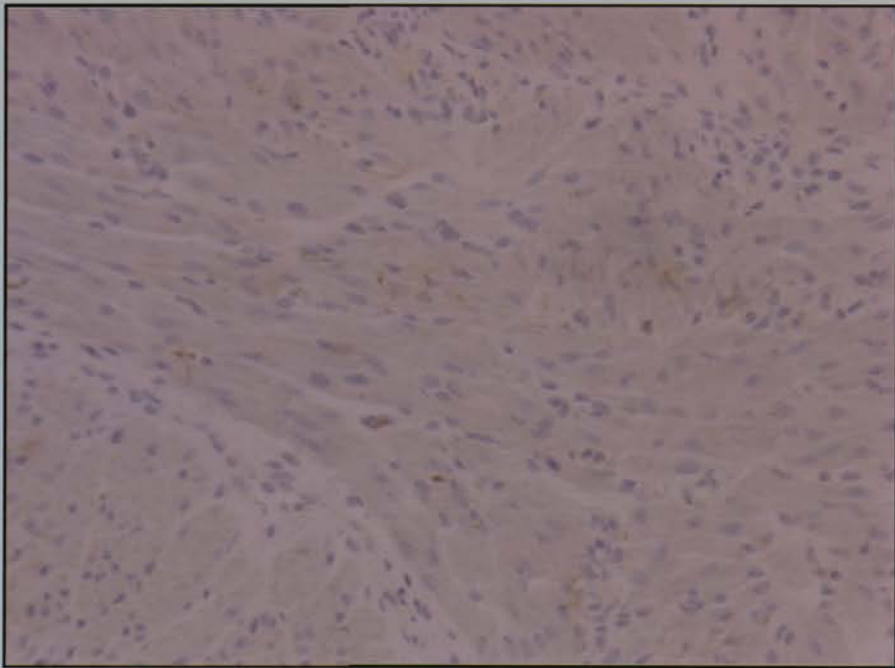


hDAF transgenic technical failure (7 hours) demonstrating no P selectin deposition (x20).

Figure 2.10. Sections of cardiac tissue stained for P selectin from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

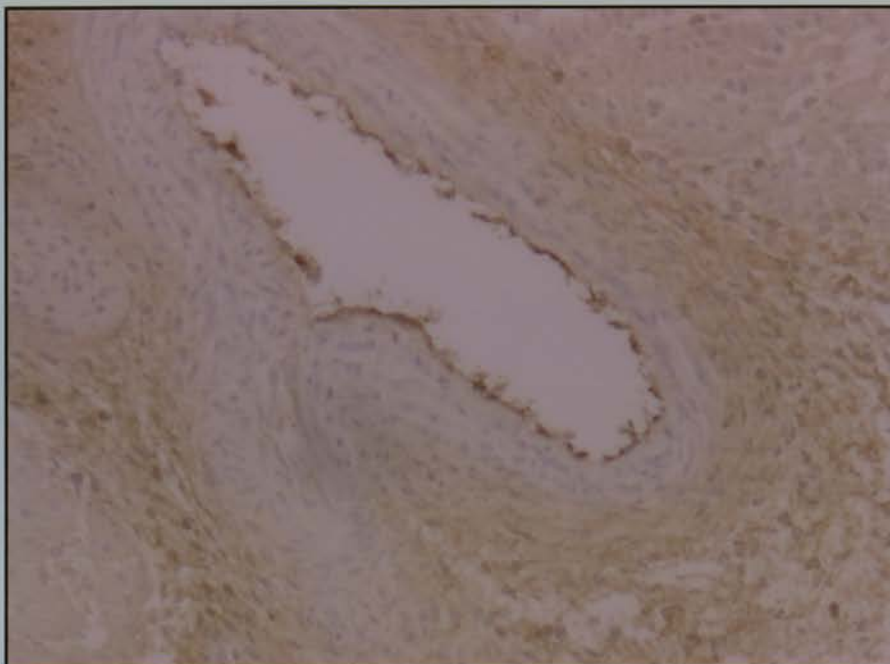


Control technical failure (6 hours) demonstrating heavy fibrin deposition (x10).

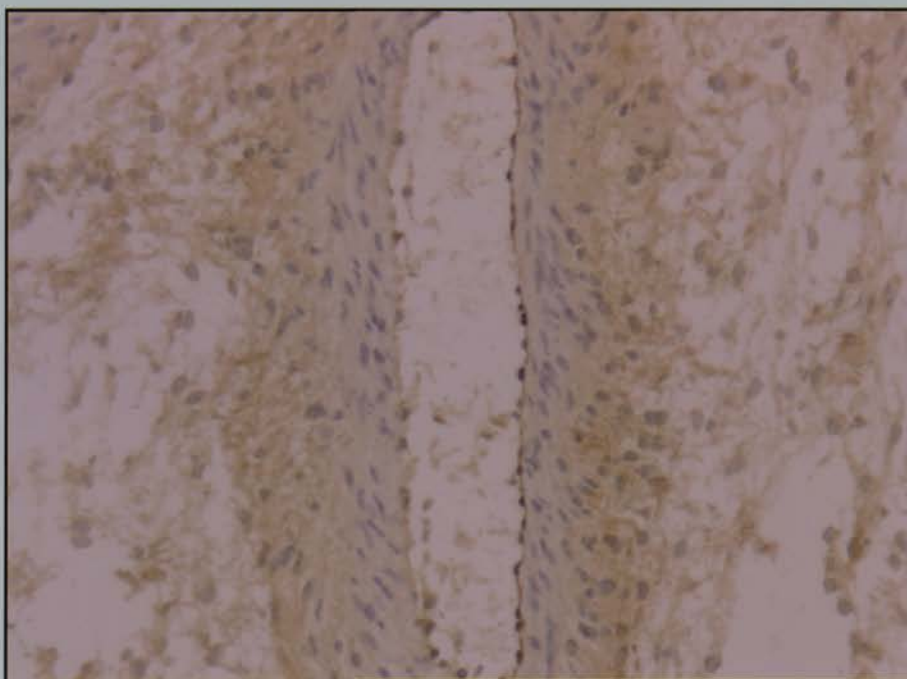


hDAF transgenic technical failure (7 hours) demonstrating slight fibrin deposition (x10).

Figure 2.11. Sections of cardiac tissue stained for fibrin deposition from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

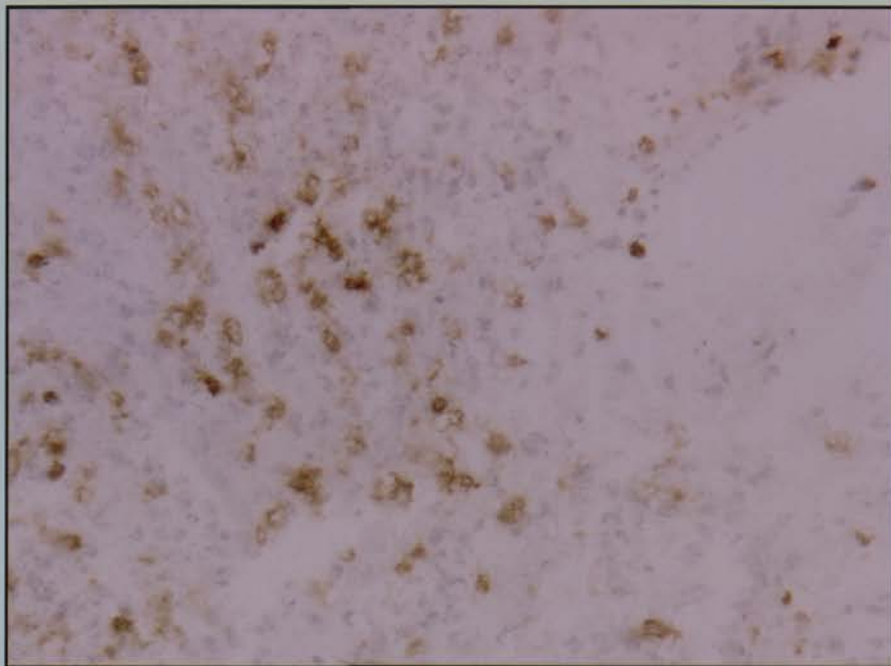


Control technical failure (6 hours) demonstrating heavy IgG binding to endothelial cells (x20).

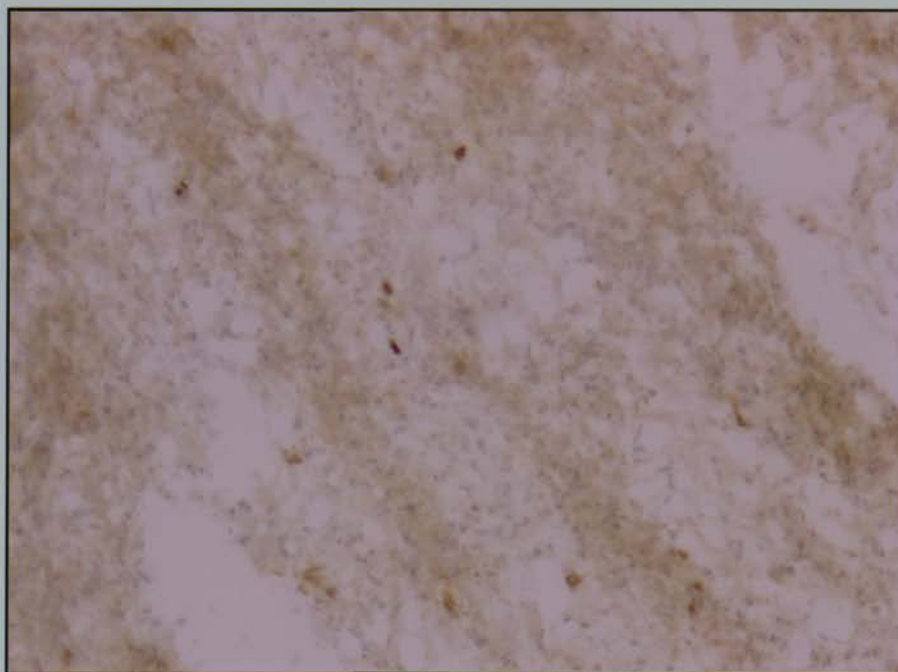


hDAF transgenic technical failure (7 hours) demonstrating moderate IgG binding to endothelial cells (x20).

Figure 2.12. Sections of cardiac tissue stained for IgG from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

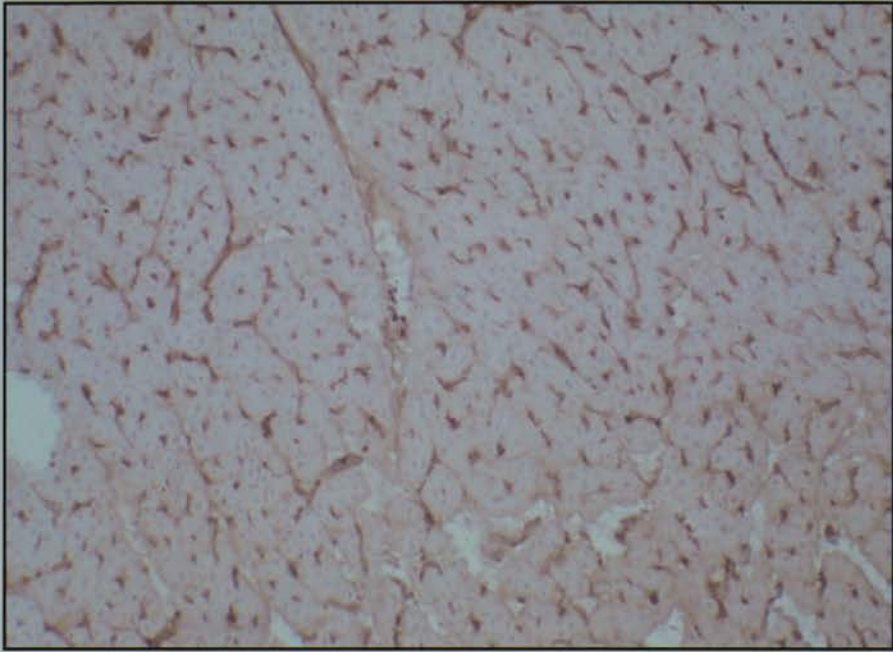


Long-surviving control heart (101 hours) demonstrating 200 CD8 T cells per high power field (x20).

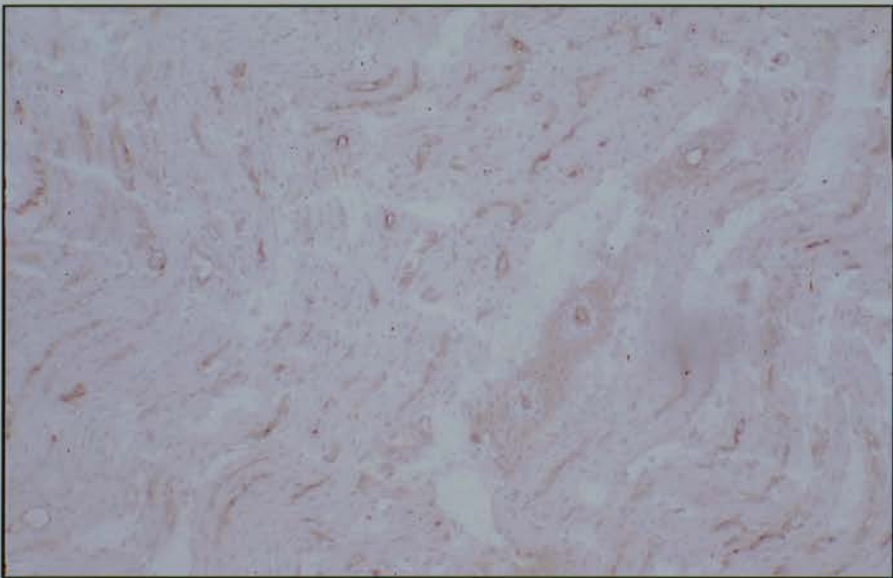


hDAF transgenic heart (97 hours) demonstrating 17 CD8 T cells per high power field (x10).

Figure 2.13. Sections of cardiac tissue stained for CD8 T cells from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.



Hyperacute rejecting control heart (6 hours) demonstrating heavy (4+) gal α gal staining(x10).



Long-surviving control heart (82 hours) demonstrating moderate (2+) gal α gal staining(x10).

Figure 2.14. Sections of cardiac tissue stained for galactose α 1-3 galactose from pig hearts transplanted heterotopically into non-immunosuppressed cynomolgus monkeys.

Discussion

Heterotopic abdominal heart transplantation from neonatal pig to cynomolgus monkey is an exacting surgical technique and in this non-immunosuppressed model there were six grafts which failed for technical reasons. However, this allowed us to collect and analyse data on control and transgenic organs failing at similar time points and so allowed us to make some important observations (see later). Of the remaining 19 technically successful operations carried out ten were performed using control (normal) pigs, eight using pigs heterozygous for the hDAF transgene and one homozygous for the hDAF transgene.

None of the transgenic grafts were hyperacutely rejected and the heterozygous transgenics had a median beating time of 5.1 days. The single homozygous transgenic had a beating time of 5.5 days. All transgenic grafts failed due to AVR.⁸¹ To our surprise, half of the control grafts were hyperacutely rejected, with a median beating time of 58 minutes and half failed due to AVR with a median beating time of 3.25 days. So it would appear that the hDAF transgene protects the xenograft against HAR and indeed that homozygosity for the hDAF transgene may confer additional protection over heterozygosity.

But, why should some normal pig hearts not undergo HAR when transplanted into the cynomolgus monkey? Analysis of the total APAbs levels, anti- α gal IgG and IgM levels and histology results may help to explain this unexpected observation (see below). Does the level of preformed natural total APAbs influence the outcome and what influence does the elicited total anti-pig antibody level have on graft function?

In most recipients there was a high preformed natural antibody level prior to transplantation which fell immediately following transplantation and this was presumably due to immunoabsorption onto the graft. It is reasonable to suppose that such binding of antibody to antigen may, under normal circumstances trigger HAR by the classical pathway of complement activation. Although there was no statistically

significant difference in the levels of preformed total APAb level in any of the groups, the mean level in the hyperacutely-rejecting controls was 2149 compared with a mean level for the long-surviving controls of 1376 and a mean level of 518 for the heterozygote transgenics. The preformed total APAb data for the transgenic group did not follow a normal distribution whereas those for the controls did, and so a direct statistical comparison cannot be made for this data set. There was however, no correlation between the preformed natural total APAb level and the graft survival time. In general, the elicited total APAb level remained low until about the third postoperative day when it started to rise rapidly resulting in graft loss in all cases by the fifth postoperative day. At the time of graft loss due to AVR the mean elicited total APAb level was 3260 ± 1836 in the control animals and 5629 ± 1242 in the transgenics, suggesting that the expression of hDAF confers on the graft some additional protection against the APAb.

In both groups the pre and post-transplant AAG IgG levels remained fairly constant suggesting that it was neither AAG IgG which was immunoabsorbed onto the graft nor was the elicited total antibody response due to AAG IgG. In contrast, the AAG IgM levels both pre and post-transplant did indeed mirror the total APAb levels suggesting that it was at least in part AAG IgM which bound to the xenograft at the time of transplantation and that it was AAG IgM which may be responsible for graft damage at the time of transplantation or indeed the triggering of HAR. In those grafts which were not hyperacutely rejected the elicited antibody response from day three post-transplant onwards appeared in part to be due to AAG IgM. Again, as with the level of total APAb pre transplant there was also a difference in the pre transplant level of AAG IgM in the transgenics and the long-surviving controls. The mean pretransplant level of preformed AAG IgM in the transgenics was lower at $11.0 \pm 4.1 \mu\text{g/ml}$ than for the long-surviving controls at $24.7 \pm 14.5 \mu\text{g/ml}$. This difference was not however statistically significant. Although these levels did not appear to affect initial graft function it raises

the possibility of some initial damage affecting later graft outcome. In the long-surviving controls the mean AAG IgM level at the time of sacrifice was $28.7 \pm 4.2 \mu\text{g/ml}$ and in the transgenic was $88.5 \pm 40.4 \mu\text{g/ml}$. This difference was statistically significant at the level $p < 0.01$.

It would appear from these results that the rise in total APAb level seen prior to rejection of the grafts was at least in part due to a rise in AAG IgM and that the expression of the transgene confers some resistance to antibody mediated AVR. Histological analysis demonstrated the classical features of HAR in half of the control grafts - widespread graft thrombosis, fibrin deposition, endothelial disruption, diffuse haemorrhage, oedema and an aggressive inflammatory cell infiltrate. These features were also seen in the transgenic grafts. The only non-immunohistological parameter for which there was a statistically significant difference between these two groups was myocyte damage, the median score being 0 in the heterozygous transgenic group and 3 in the hyperacute rejecting control group ($p < 0.05$). However, when comparing these histological parameters one must take into consideration that we were comparing control hearts with a median beating time of 0.96 hours with transgenic grafts with a median beating time of 5.1 days. To compare these histological parameters at similar time points for the two groups we can turn to the six transplants which failed for technical reasons. Of the six transplants three were controls with a median beating time of 5.5 hours and three were transgenics with a median beating time of 3 hours. Every histological parameter indicated significantly more graft damage in the control hearts.

Immunohistological analysis of technical failure control hearts compared with technical failure transgenic hearts demonstrated no difference in the amount of endothelial C4 deposition but significantly more endothelial C3 and C9 deposition in the control grafts. This pattern was exactly as would be predicted from the mode of action of hDAF since this complement regulator acts at the point of formation of the C3 convertases C4b2a and C3bBb (See chapter 1).

There was also significantly more P selectin expression in the technical failure control group suggesting endothelial cell activation even at this early stage, with a score of 2 in the controls and 0 in the transgenics. There was also evidence of greater activation of the coagulation cascade in the control group with significantly more fibrin deposition in the control grafts and slightly more Von Willebrand's factor deposition in the controls, although this difference was not statistically significant.

Of the technically successful control grafts 50% were not hyperacutely rejected and underwent AVR after a surprisingly long period and although most histological parameters at the time of rejection were similar in both the long-surviving controls and the transgenic grafts there was significantly more vasculitis, platelet thrombi, diffuse haemorrhage and endocardial permeability in the long-surviving control group. There was also significantly more P selectin expression in the long-surviving controls and a significantly greater cellular infiltrate of CD-4 T cells, CD-8 T cells, CD-19 B cells, CD-68 macrophages and tissue neutrophils.

There was a significant difference in the amount of GSL.1 binding on the endothelium of the grafts, with a median score of 3.5 for the transgenic group and 2 for the long-surviving control group. It is this latter finding which may help to explain why these control animals survived far in excess of what was expected. The relative lack of antigenicity in this group may be of great importance. Indeed when compared with those control grafts which were hyperacutely rejected there was a statistically significant difference in the amount of GSL 1. binding, the score being 3 for the hyperacutely rejecting controls. GSL.1 binds specifically to galactose α 1-3 galactose on endothelium of tissues and it this sugar residue which is thought to represent the major antigenic epitope in xenotransplantation.

Histological analysis of the single homozygous graft demonstrated that there was less endocardial adherence, endocardial disruption and oedema in the homozygous heart, compared with the heterozygous hearts, but surprisingly more myocyte damage,

myocytolysis and diffuse haemorrhage. There was no difference in any of the other histological or immunohistological parameters.

Finally, these results of heterotopic transplantation of normal and hDAF transgenic pig hearts into non-immunosuppressed cynomolgus monkeys suggest that hDAF does indeed confer protection against HAR. However, other factors may also be playing a part and the antigenicity of the graft as measured by GSL1 binding is probably important. In addition, it would appear that the level of preformed antibodies present in the recipient at the time of transplantation, in the form of total anti-pig antibodies and AAG IgM antibodies, may also influence graft function and the presence of hDAF on the graft confers some degree of protection against the elicited total APAb and the elicited AAG IgM antibody mediated AVR.

Chapter Three

Immunosuppressive strategies in pig-to-cynomolgus monkey heterotopic cardiac xenotransplantation

Introduction

We have demonstrated in chapter two that hDAF transgenic pig hearts are not hyperacutely rejected when transplanted into the cynomolgus monkey but that such grafts stimulate a strong anti-pig antibody response which results in graft loss due to acute vascular rejection. It is the aim of the experiments in this chapter to demonstrate how the anti-pig antibody (APAb) response can be modulated with immunosuppressive agents and therefore prolong graft survival. In this study we have chosen a simple and clinically applicable regimen consisting of cyclosporin A (CyA), cyclophosphamide (CyP) and steroids.

During the last fifty years, many immunosuppressive agents and modalities have been used in the field of transplantation. In the 1950s, total body irradiation was shown to induce immunosuppression and prolong survival of renal transplants in dogs. It was first used in a human renal transplant recipient in 1958, but proved to be too toxic for routine immunosuppression.⁸² The development of immunosuppressive drugs in the 1950s provided some hope that reducing the risk of rejection might be possible without causing the severe toxicity associated with irradiation. One of the first immunosuppressive agents to be identified was 6-mercaptopurine.⁸³ It was first given to the recipient of a human renal transplant in 1959.⁸² Shortly following this azathioprine was used in a patient in 1961.⁸⁴

The other principal agent to emerge at this time was the corticosteroid, prednisolone, which was shown in 1963 to improve kidney graft survival in dogs.⁸⁵ It is now known that corticosteroids interfere with T cell proliferation through their ability to block activation of the interleukin-1 and interleukin-6 genes.⁸⁶ As a result, azathioprine and prednisolone became the standard immunosuppressive agents throughout the 1960s and 1970s.

Cyclophosphamide has also been used in clinical practice for its immunosuppressive effect in both arthritis⁸⁷ and organ transplantation.⁸⁸

CyP is an alkylating agent which acts by disrupting cell growth, mitotic activity and differentiation by cross-linking DNA strands. It therefore interferes with the normal cell division in all rapidly proliferating tissues. It is however, potentially toxic with a wide range of side-effects in humans, the most serious being bone-marrow suppression, haemorrhagic cystitis, pneumonitis, pulmonary fibrosis and cardiotoxicity, the latter three effects being thankfully rare. Gastrointestinal upset although usually less threatening is also common.⁸⁹

There is some in vitro evidence to suggest that CyP, being a cell cycle-specific agent, is both toxic for inter mitotic and replicating cells and that it should be most effective if therapy coincides with the period of extensive lymphoproliferation i.e. commenced shortly after the immunological stimulus.⁹⁰

Various other methods have been investigated. X-ray therapy over the transplant itself appeared to prolong survival, while splenectomy and thymectomy reduced the body lymphocyte pool.⁹¹ Antilymphocyte globulin⁹¹ was also developed as was thoracic duct drainage.⁹²

However, it wasn't until 1973 that cyclosporin A was purified from the fungi *Cylindrocarpon lucidum* and *Tolypocladium inflatum* and found to have a marked effect on both humoral and cell-mediated immunity.⁹³ Further experiments showed that CyA was able to suppress the rejection of heart allografts in rats⁹⁴ and renal allografts in dogs.⁹⁵

A number of subsequent studies have shown that the predominant action of CyA is on helper T lymphocytes, suppressing the production of lymphokines, particularly interleukin-2.⁹⁶

FK506⁹⁷ is a macrolactam isolated from *Streptomyces tsukubaensis* which blocks IL-2 gene transcription and therefore has a similar effect to CyA.

Although standard immunosuppressive agents have proven highly effective for allotransplantation, they are relatively impotent, when used alone, in preventing

xenograft rejection. Suppression of anti-species antibodies is the main aim of immunosuppression.

In this study CyP was chosen as the anti-B cell agent as prolonged survival of hamster-to-rat heart xenografts has been demonstrated when CyP was combined with Cyclosporin A.⁹⁸ Methotrexate has also been shown to be effective in this model.⁹⁹

In concordant primate xenotransplantation models such as cynomolgus monkey-to-baboon heterotopic heart transplantation the simple combination of CyA and prednisolone has resulted in a mean graft survival of 77 days.¹⁰⁰ Total lymphoid irradiation in combination with CyA and prednisolone has resulted in survival of cynomolgus monkey hearts transplanted heterotopically into baboons for greater than 480 days.¹⁰¹

The combination of CyA, prednisolone, FK506 and methotrexate has resulted in graft survival in an orthotopic heart transplantation model from rhesus monkey to baboon of up to 500 days.¹⁰²

Predictably, however, discordant pig-to-primate xenotransplantation has been less successful. Pig hearts transplanted heterotopically into Japanese monkeys, immunosuppressed with FK506 and nafamstat mesilate (complement inhibitor), in combination with splenectomy and double filtration plasmapheresis resulted in graft survival of only 270 minutes.¹⁰³

The combination of cobra venom factor, splenectomy, CyA, prednisolone, CyP and methotrexate has resulted in pig-to-baboon heterotopic cardiac xenograft survival of 25 days.¹⁰⁴

Continuous infusion of soluble complement receptor type 1 (sCR1) and immunosuppression of cynomolgus monkeys with CyA, CyP and steroids resulted in heterotopic pig cardiac xenograft survival for up to 32 days, the monkey being sacrificed due to systemic infection.¹⁰⁵

Materials and Methods

Donor and recipient selection

The criteria used for donor and recipient selection and the code of practise followed are described on pages 38-39.

In addition all monkeys selected as recipients were screened for total anti-pig red blood cell haemolytic antibodies and only those with antibody levels greater than a pool of human sera were used as recipients so that the transgene would be tested to a degree which would attempt to reproduce the clinical pig-to-human situation.

Donor and recipient operations and postoperative management

A full description of the donor and recipient operations and the postoperative management is described on pages 40-43.

Immunosuppression

All recipient cynomolgus monkeys were immunosuppressed with a combination of CyA, steroids and CyP, the doses and schedule being summarised in table 3.1 and described in greater detail below.

Cyclosporin A

All animals received CyA, commencing on the day prior to transplantation, to achieve trough levels of 400 ng/ml. CyA was administered for the first 5 days intramuscularly (Sandimmun) and thereafter orally (Neoral).

Steroids

Methyl prednisolone treatment was commenced at 1mg/kg/day reducing to 0.2mg/kg/day by day 18.

Cyclophosphamide

Two CyP regimens were used:

Group 1 (n=10)- CyP was given on the preoperative day at 40mg/kg, operative day at 20mg/kg and second postoperative day at 40mg/kg and alternate days thereafter, tailored to the white cell count (wcc) and the anti-pig antibody (APAb) levels.

Group 2 (n=5)- no CyP until day two at 40mg/kg and day three at 20mg/kg and thereafter only if indicated by a rising APAb level.

All control animals (n=5) were commenced on the group 1 CyP regimen.

CyP was temporarily discontinued if the total wcc fell below 2×10^6 cells /ml and was usually increased if the APAb level started to climb.

Cyclosporin A	Steroids	Group 1 Cyclophosphamide	Group 2 Cyclophosphamide
25mg/kg i.m. for first 5 days and orally thereafter to achieve plasma levels of 400ng/ml	Methylprednisolone 1mg/kg i.v. at surgery and oral prednisolone daily thereafter reducing by 0.05mg/kg/day to 0.2mg/kg by day 18	Day -1, 40mg/kg Day 0, 20mg/ kg Day 2, 40mg/kg 20-40mg/kg alternate days thereafter as dictated by APAb and wcc	Day 2 40 mg/kg, Day 3 20mg/kg 20-40mg/kg alternate days thereafter as dictated by APAb and wcc

Table 3.1. Immunosuppressive regimen. i.m.=intramuscular, i.v.=intravenous, APAb=anti-pig haemolytic red blood cell antibodies, wcc=white cell count.

Blood sampling and monitoring

Blood was sampled regularly for total anti-pig haemolytic antibody (APAb) levels (as described in chapter two), trough CyA levels (thin-layer chromatography, Papworth Hospital), full blood count and differential wcc (Bayer-Technicon haematology analyser), electrolytes and creatinine (Hitachi 737 clinical chemistry analyser) and the immunosuppression was tailored accordingly (table 3.1).

Diagnosis of xenograft rejection

Rejection was defined as the absence of palpable cardiac xenograft pulsation. This was then confirmed histologically.

Histology

Donor

All donor ear clippings were scored for hDAF expression as described in chapter 2.

Recipient

All explanted hearts were examined histologically by staining with haematoxylin and eosin (H+E) and immunochemistry for all parameters as described in chapter two.

Bone marrow

Bone marrow from selected recipients was stained with Giemsa and examined for the presence of leukoerythroblastic precursors.

Bowel

Small and large bowel from those recipients developing diarrhoea was stained with H+E and carefully examined to elucidate the aetiology for the diarrhoea.

Statistical analysis

Analysis of survival data was performed using permutation tests on the ranked data (Huntingdon Life Sciences Ltd, Huntingdon). Parametric data was analysed using the two-tailed t test assuming unequal variances (Excel version 4.0). Non-parametric data was analysed using the Mann-Whitney test (SPSS 7.5 for windows).

Results

Fifteen heterozygous transgenic and five control hearts were transplanted heterotopically into the abdomen of immunosuppressed cynomolgus monkeys. Of the transgenic hearts ten were immunosuppressed with the high CyP regimen (group 1) and five were immunosuppressed with the low CyP regimen (group 2). All control hearts were immunosuppressed with the high CyP regimen. The donor and recipient parameters can be seen in table 3.2.

Parameter	Group 1	Group 2	Controls
Donor Weight (mean±SD)	2.74±0.6 kg	3.34±0.88 kg	2.97±0.79 kg
Recipient Weight (mean±SD)	2.35±0.4 kg	3.06±0.26 kg	2.44±0.16 kg
Ischaemic Time (mean±SD)	1.31±0.26 hours	1.72±0.44 hours	1.63±0.18 hours
Recipient Country of Origin	Mauritius	Mauritius	Mauritius

Table 3.2. Immunosuppressed donor /recipient parameters.

Note that there was no significant difference in the control, high CyP or low CyP transgenic groups in the mean donor weight or recipient country of origin. However, the group 2 (low CyP) recipients had a slightly greater recipient mean body weight than either the group 1 (high CyP transgenic group) or the controls. This reached statistical significance at $p < 0.01$. The mean ischaemic time was also significantly longer ($p < 0.05$) for the control grafts when compared with group 1 grafts, but not when compared with group 2 grafts. The outcome can be seen in table 3.3.

The hDAF transgenic hearts in the high CyP group had a median beating time of 40 days (range 6-62 days) compared with 9 days (range 8-34 days) for those in the low CyP group (figure 3.1). This difference was statistically significant ($p < 0.05$). The control grafts had a median beating time of 55 minutes (range 2-180 minutes). The difference between the control grafts and the high CyP transgenics was statistically highly significant ($p < 0.001$). The difference between the control grafts and the low CyP transgenics was also statistically significant ($p < 0.05$).

Recipient	Genetics	Immunosuppression	Beating Time	Histology	Cause of Failure
W142	T	High CyP	6 days	AVR	Rejection
W141	T	High CyP	34 days	NER (E)	Diarrhoea
W140	T	High CyP	35 days	NER (E)	Diarrhoea
W326	T	High CyP	45 days	NER (E)	GI Bleed
W143	T	High CyP	54 days	NER	Cardiomyopathy
W323	T	High CyP	28 days	NER (E)	Diarrhoea
W374	T	High CyP	8 days	NER (E)	Peritonitis
W328	T	High CyP	57 days	NER (E)	Diarrhoea
W333	T	High CyP	55 days	NER	Not identified
W341	T	High CyP	61 days	AVR	Rejection
W544	T	Low CyP	34 days	AVR	Rejection
W554	T	Low CyP	10 days	AVR	Rejection
W112	T	Low CyP	8 days	AVR	Rejection
W445	T	Low CyP	9 days	AVR	Rejection
W459	T	Low CyP	8 days	AVR	Rejection
W428	C	High CyP	2 minutes	HAR	Rejection
W434	C	High CyP	35 minutes	HAR	Rejection
W427	C	High CyP	55 minutes	HAR	Rejection
W430	C	High CyP	180 minutes	HAR	Rejection
W431	C	High CyP	60 minutes	HAR	Rejection

Table 3.3. Outcome of immunosuppressed pig-to-cynomolgus monkey cardiac xenografts. T=transgenic, C=control, CyP=cyclophosphamide, AVR=acute vascular rejection, NER=no evidence of rejection, (E)=euthanased.

All control hearts were hyperacutely rejected. Only two hearts in the high CyP group were rejected, on days 6 and 62: all other hearts showed no evidence of rejection. Both rejected hearts demonstrated AVR histologically, but the heart which was rejected on day six was shown histologically to express very little hDAF on it's endothelium. This was also the first cynomolgus recipient to be immunosuppressed and it was clear that cynomolgus monkeys do not absorb CyA and CyP with the same profile as humans. As a result inadequate doses of drugs were administered to this recipient and the heart was rejected on day six. Of the remaining eight animals in the high CyP group, one heart failed due to a cardiomyopathy on day 54 and one heart stopped beating on day 55 but there was no evidence of rejection and no cause for it's failure identified. The

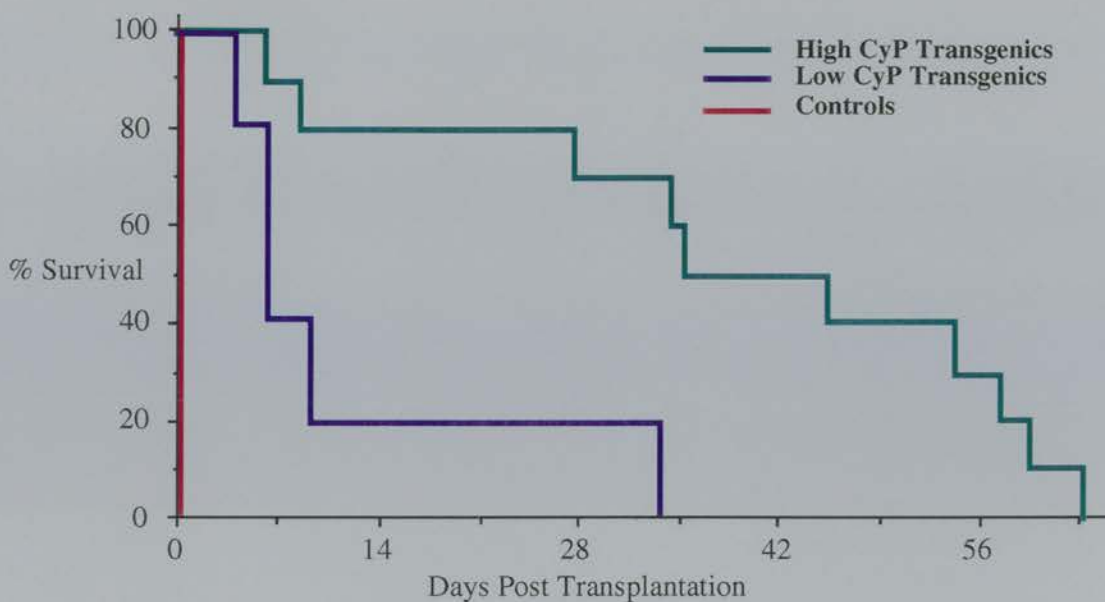


Figure 3.1. Survival of control and transgenic pig hearts in immunosuppressed cynomolgus monkeys.

other six animals were sacrificed with beating cardiac xenografts in compliance with Home Office Animal Welfare guidelines as a result of gastrointestinal complications. One recipient was sacrificed on day eight due to peritonitis secondary to an intra abdominal abscess adjacent to the graft, one recipient was sacrificed following a gastrointestinal bleed secondary to colonic polyps and the remaining four recipients were sacrificed with intractable diarrhoea. No infective agent was identified by repeated blood and stool culture. All hDAF transgenic hearts in the low CyP group underwent AVR. The survival and outcome for the recipients of transgenic pig hearts is summarised in table 3.3.

Immunosuppression

Cyclophosphamide dose

The mean CyP dose received by the recipients in group 1 perioperatively and during the first nine days postoperatively was $17.9 \pm 3.6 \text{ mg/kg/day}$ compared with $10.3 \pm 1.9 \text{ mg/kg/day}$ in group 2 ($p < 0.001$). This represented a 45% reduction in dose

(figure 3.2). Beyond day nine little data exists for group 2. However, the mean dose received by group 1 recipients from the ninth postoperative day onwards was $6.4 \pm 2.0 \text{ mg/kg/day}$ compared with 4.4 mg/kg/day for W544, the only group 2 recipient surviving beyond day nine. Hence, the two methods of CyP dosing resulted in a significantly greater amount of CyP being administered perioperatively, and in the early postoperative period, in group 1 but smaller and comparable doses being administered to both groups in the later postoperative period.

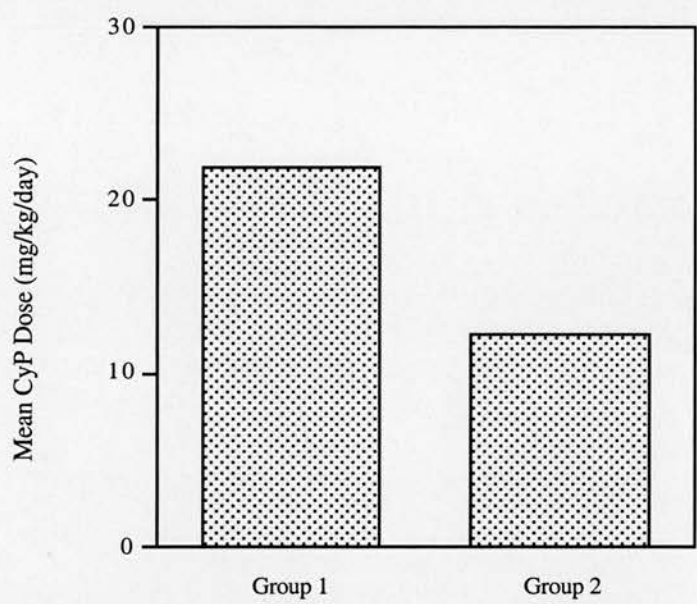


Figure 3.2. Mean cyclophosphamide dose received by recipients during the first nine days following transplantation.

Cyclosporin A dosing and levels

CyA was administered intramuscularly at a dose of 25 mg/kg for the first five days in both groups and thereafter orally in two divided doses with the aim of achieving plasma CyA levels of 400 ng/ml . In group 1 the mean plasma CyA level achieved was $554 \pm 70.7 \text{ ng/ml}$ with a mean total daily CyA dose required to achieve this level of $122 \pm 19.9 \text{ mg/kg}$ (range $40\text{-}350 \text{ mg/kg}$).

In group 2 the mean plasma CyA level achieved was 876 ± 291 ng/ml with a mean total daily CyA dose required to achieve this level of 109 ± 9.1 mg/kg (range 60-155 mg/kg). There was no significant difference in the CyA dose administered in either group and although the CyA level achieved in group 2 was greater this did not quite reach statistical significance ($p=0.07$).

Creatinine levels

Plasma creatinine levels remained within normal limits (0.5-0.8 mg/dl) for all recipient cynomolgus monkeys receiving CyA in both groups.

Total white cell count

The mean total wcc fell to $2.1 \pm 0.83 \times 10^6$ /ml (normal range $6-17.1 \times 10^6$ /ml) by the eighth postoperative day in group 1 but only fell to its lowest point of $4.7 \pm 0.91 \times 10^6$ /ml on the seventh postoperative day in group 2 (figure 3.3). Note that the mean total wcc was slightly higher in the group 2 recipients at the start of the experiment. Despite this, the pattern of change in the mean total wcc was broadly similar in both groups during the first 15 days.

The sharp fall in mean total wcc seen on day three in group 1 wasn't seen in group 2 until day four. In both groups the early reduction in CyP dose resulted in a slight recovery of the wcc on days four and five in groups 1 and 2 respectively. This was also associated with a concomitant rise in total APAb levels in both groups and therefore additional doses of CyP (see figures 3.11 and 3.12) were administered with a resultant further fall in mean total wcc. Beyond day ten the data for group 2 relates to only one recipient, i.e. W544, compared with eight recipients for group 1, hence a detailed comparison cannot be drawn during this period.

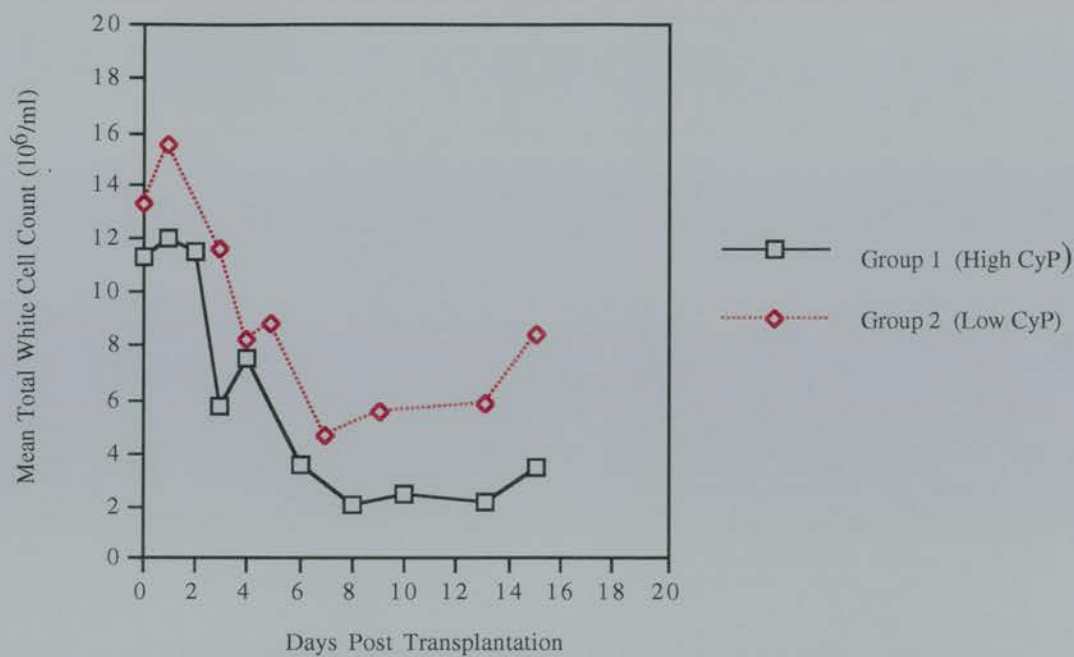


Figure 3.3. Mean total white cell count in high and low cyclophosphamide groups during the first 15 days following transplantation.

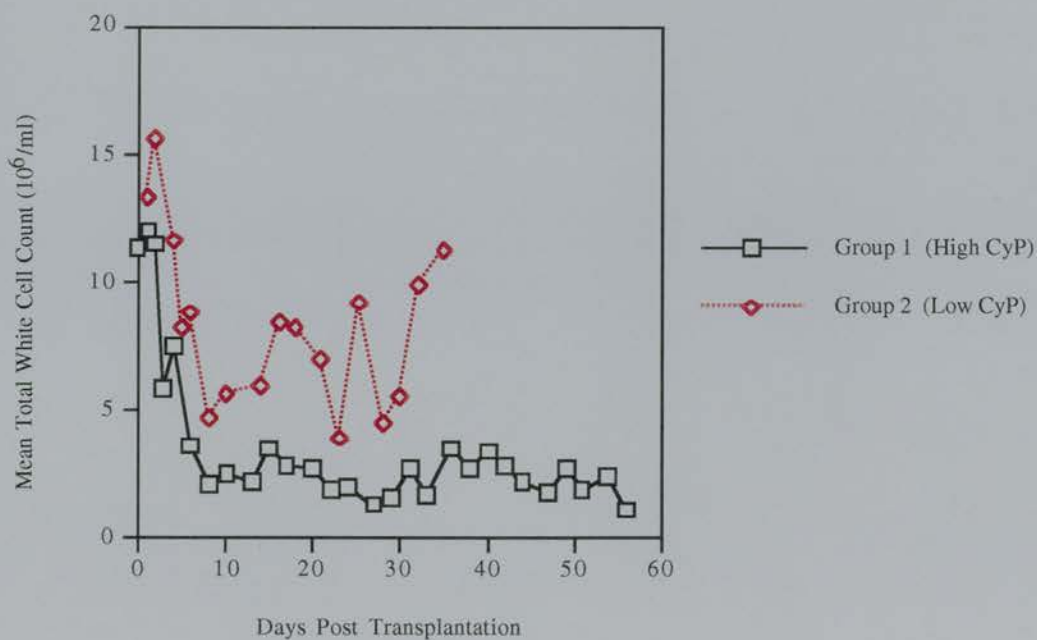


Figure 3.4. Mean total white cell count in high and low cyclophosphamide groups following transplantation.

However, we can see from figure 3.4 that the mean total wcc for group 1 remained consistently between 2 to 4×10^6 cells/ml, whereas the total wcc for the single recipient in group 2 varied widely from 3 to 12×10^6 cells/ml.

Lymphocyte count

We can see from figure 3.5 that the mean lymphocyte count in group 1 fell from a preoperative level of 7×10^6 /ml (normal range $3.5\text{--}12.5 \times 10^6$ /ml) to 2×10^6 /ml on the first postoperative day. The mean lymphocyte count in group 2, however, started at a preoperative level of 4.2×10^6 /ml and remained about this level for the first few days and didn't fall to 2×10^6 /ml until the seventh postoperative day.

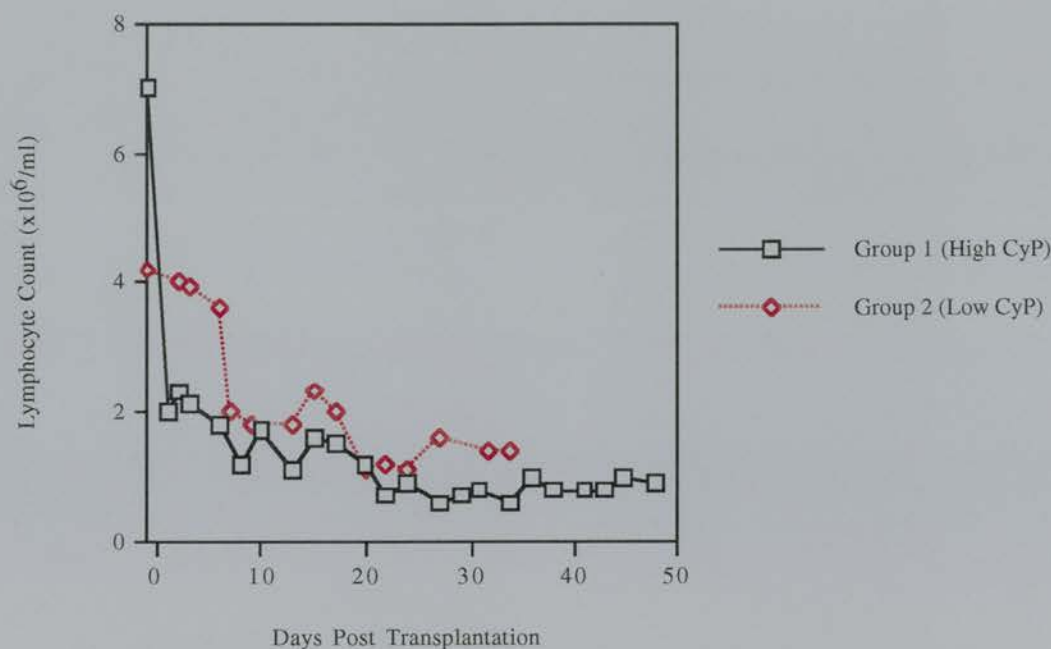


Figure 3.5. Mean lymphocyte count in high and low cyclophosphamide groups.

In group 1 the mean lymphocyte count slowly fell from 2×10^6 /ml to 1×10^6 /ml during the first 20 postoperative days and then fluctuated between 0.5 to 1×10^6 /ml thereafter. In group 2 there was a greater fluctuation in the downward trend beyond the seventh postoperative day between 1.0×10^6 /ml and 2.5×10^6 /ml. The level in group 2 remained

higher than in group 1 for most of the time. Beyond day ten the data for group 2 relates to only one recipient, i.e.W544, compared with eight recipients for group 1, hence a detailed comparison cannot be drawn during this period.

Neutrophil count

We can see from figure 3.6 that there was a marked rise in the mean neutrophil counts in both groups immediately following transplantation (normal range $0.9\text{-}7.5 \times 10^6/\text{ml}$). This was followed in both groups by a sharp fall in the mean neutrophil count, followed by a more gradual fall to the tenth postoperative day. Beyond day 10 the mean neutrophil count was seen to vary from $0.5 \times 10^6/\text{ml}$ to $3.0 \times 10^6/\text{ml}$ in the group 1 recipients but there is no mean data available for comparison in group 2.

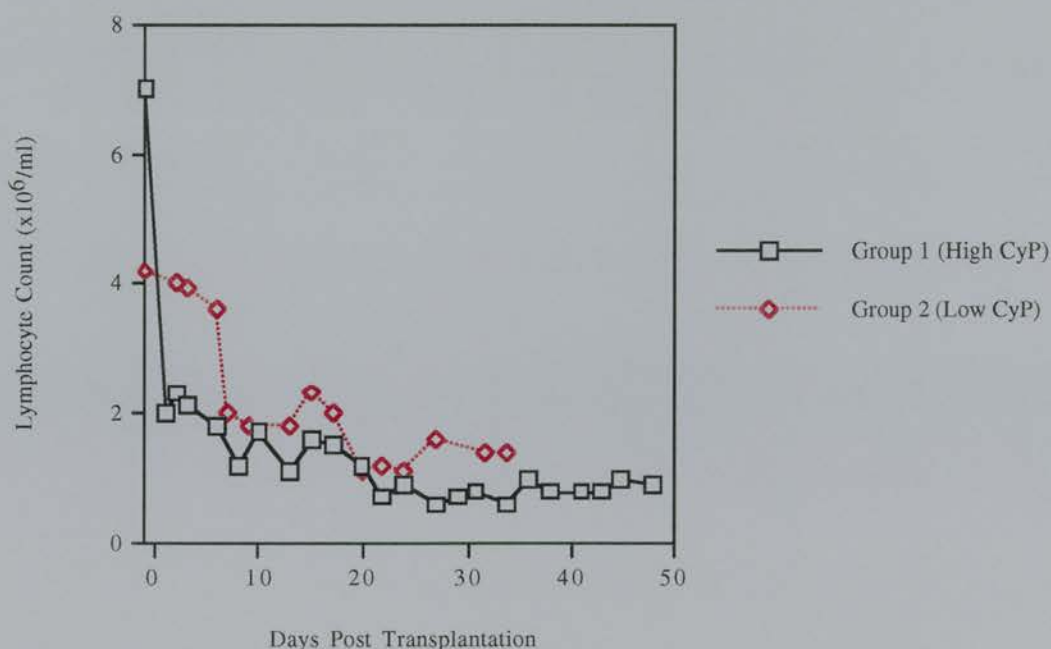


Figure 3.6. Mean neutrophil count in high and low cyclophosphamide groups.

Haemoglobin concentration

The postoperative haemoglobin (Hb) concentration (normal range 10.2-13.7g/dl) fell by similar amounts in both groups (figure 3.7). There was an initial sharp fall noted by

the first postoperative day followed by a steady decline in Hb concentration thereafter. There was a mean fall of $4.25 \pm 1.5 \text{ g/dl}$ ($26 \pm 11\%$ of preoperative Hb) in group 1 and $3.5 \pm 1.9 \text{ g/dl}$ ($27.4 \pm 14\%$ of preoperative Hb) in group 2 by the ninth postoperative day. This difference was not significant. The pattern of change in the mean Hb concentration was broadly similar in both groups (note the data for group 2 beyond the

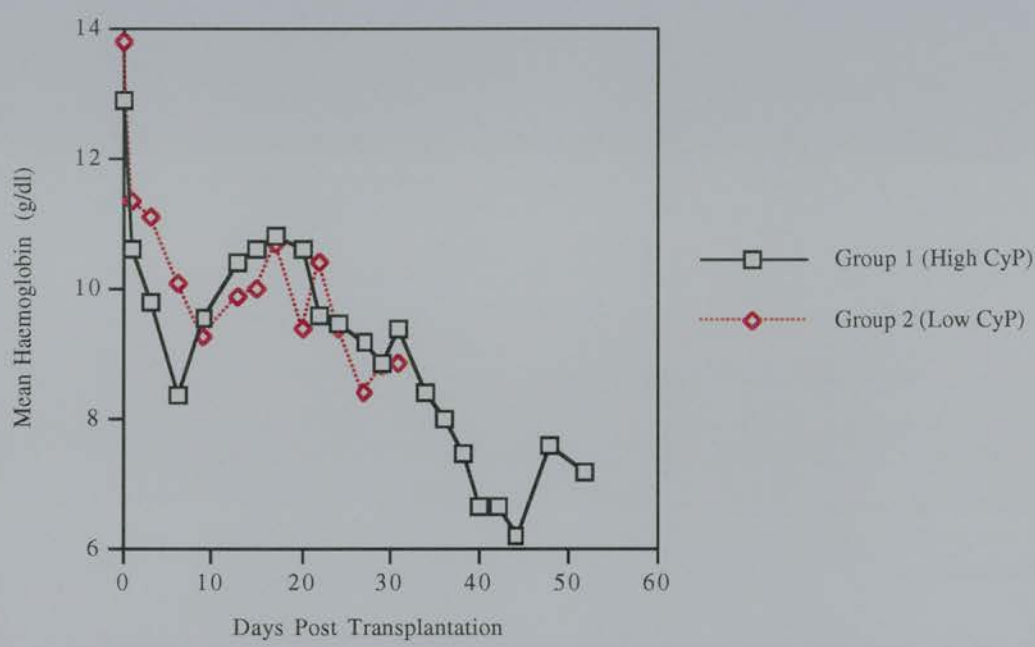


Figure 3.7. Mean haemoglobin concentration in high and low cyclophosphamide groups.

tenth postoperative day represents a single recipient). The mean Hb concentration in group 1 fell from its preoperative level of $12.9 \pm 0.7 \text{ g/dl}$ to $8.35 \pm 1.5 \text{ g/dl}$ by the sixth postoperative day. The mean Hb concentration in group 2 fell from its preoperative level of $13.8 \pm 0.4 \text{ g/dl}$ to a low point of $9.3 \pm 1.2 \text{ g/dl}$ by the ninth postoperative day. Thus although the fall in Hb concentration followed a similar pattern in both groups, there was a lag in the fall in group 2 as might be expected from the dosing pattern of CyP. Following this initial fall there was a partial recovery of the Hb concentration by the 17th postoperative day to $10.8 \pm 1.2 \text{ g/dl}$ in group 1 and 10.7 g/dl in group 2.

Beyond this point there was a steady and continuous fall in the Hb concentration in both groups (note group 2 is represented by W544 only) at a rate of 0.2g/dl/day.

Platelet count

We can see from figure 3.8 that in group 1 the mean platelet count fell (normal range $280\text{--}650\times10^6/\text{ml}$) from it's preoperative value of $341\pm29\times10^6/\text{ml}$ to $269\pm74\times10^6/\text{ml}$ on the first postoperative day and that this was then followed by a reactive thrombocytosis which reached a level of $429\pm124\times10^6/\text{ml}$ on the tenth postoperative day. Beyond the tenth postoperative day the platelet count gradually fell until it reached a level of $163\pm38\times10^6/\text{ml}$ on the 55th postoperative day.

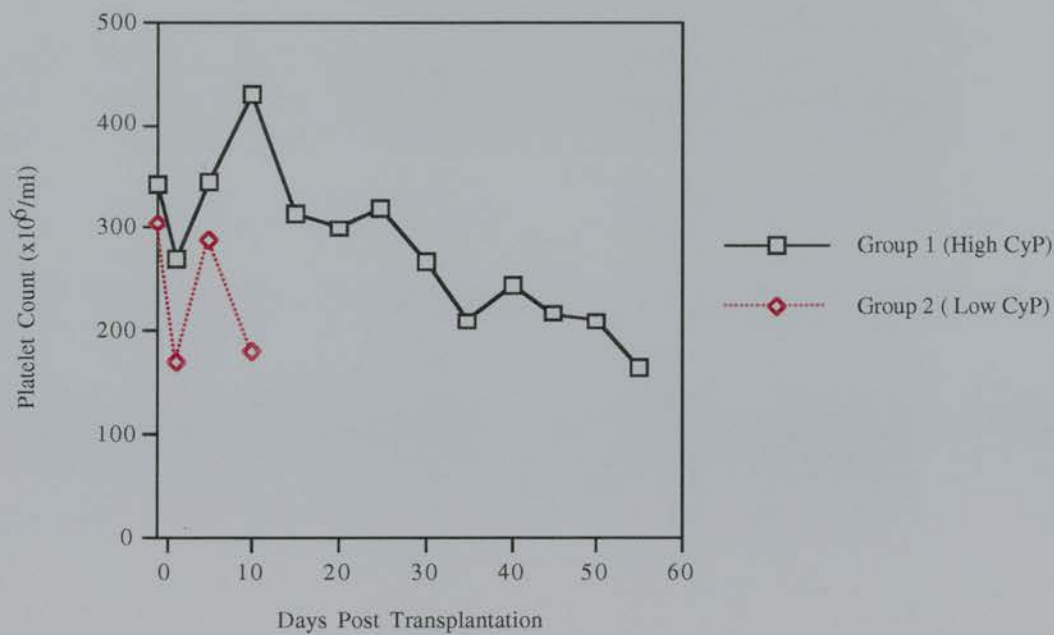


Figure 3.8. Mean platelet count in high and low cyclophosphamide groups.

In group 2 the mean platelet count fell from it's preoperative value of $305\pm46\times10^6/\text{ml}$ to $168\pm59\times10^6/\text{ml}$ on the first postoperative day and was then followed by a reactive thrombocytosis which reached a level of $286\pm112\times10^6/\text{ml}$ on the fifth postoperative day.

Unlike group 1, which had its peak platelet count on day ten, the platelet count in group 2 fell on day ten to $178 \pm 33 \times 10^6/\text{ml}$ (note that there is insufficient data in group 2 beyond day ten to draw a comparison).

Total anti-pig antibody levels

We can see in figure 3.9 that the preformed natural total APAb levels in group 1 prior to transplantation were high and that these immediately fell almost to zero following transplantation (presumably due to immunoabsorption onto the graft). Indeed the mean preformed APAb level in group 1 was 1542 ± 246 . The elicited total APAb levels, in general, remained low until about the third postoperative day, at which time they began to rise and then tended to peak by the fifth postoperative day. Beyond the fifth postoperative day there were a variable number of peaks and troughs of antibody levels. The amplitude of these peaks also varied widely. Despite the many antibody peaks, however, only two resulted in xenograft rejection i.e. that on day six which resulted in graft rejection in recipient W142 and that on day 61 which resulted in graft rejection in recipient W341.

The preformed natural total APAb levels in group 2 (figure 3.10) followed a similar pattern to those in group 1, being high (mean 1700 ± 276) and then falling almost to zero immediately following transplantation. Indeed there was no statistical difference in the levels of the pretransplant APAb levels.

In contrast, the pattern of elicited total APAb levels and their effect on graft viability in group 2 was markedly different. The levels initially remained low until about the fifth to eighth postoperative days, at which time there was again an increase in antibody levels in four out of five recipients which resulted in every case in xenograft rejection. The only recipient in group 2 in which there wasn't an antibody increase around this time was W544. In W544 the elicited total APAb level remained low until the 24th postoperative day at which time there was a small peak and a later increase in antibody

level on the 33rd postoperative day resulted in xenograft rejection. We can see in figure 3.11 that the pretransplant total APAb level in recipient W141 (group 1) fell to almost zero following transplantation and that the total wcc initially rose. Despite the pre and perioperative CyP doses, the elicited total APAb level started to rise until the eighth postoperative day, at which time the total APAb level again fell and remained suppressed until day 14. Note that in response to the rising APAb level the dose of CyP was increased to 40mg/kg given for three consecutive doses at alternate day intervals. This resulted in a falling total wcc, which by day ten was almost zero and so no further CyP was given until 14, at which time the total wcc had started to recover. CyP was then given in 20mg/kg doses on alternate days in an attempt to suppress both the total wcc and the antibody level. Despite this maintenance dose of CyP the total APAb level started to rise again on day 20 and reached a peak on day 24. In response to the rising APAb level the CyP dose was further increased on day 21 which resulted in a falling total wcc and a falling APAb level beyond day 24. The recipient was sacrificed under Home Office guidelines with a beating cardiac xenograft due to intractable diarrhoea and poor general condition.

We can see in figure 3.12 that the pretransplant total APAb level in recipient W554 (group 2) fell to almost zero following transplantation and that the total wcc initially rose in exactly the same way as with W141. However, on this occasion no pre or perioperative CyP was given, the first dose of CyP being 40mg/kg on the second postoperative day. The elicited total APAb level remained low until a modest spike on day nine resulted in xenograft rejection on day ten, despite additional CyP doses between days eight and ten, even in the face of a low total wcc.

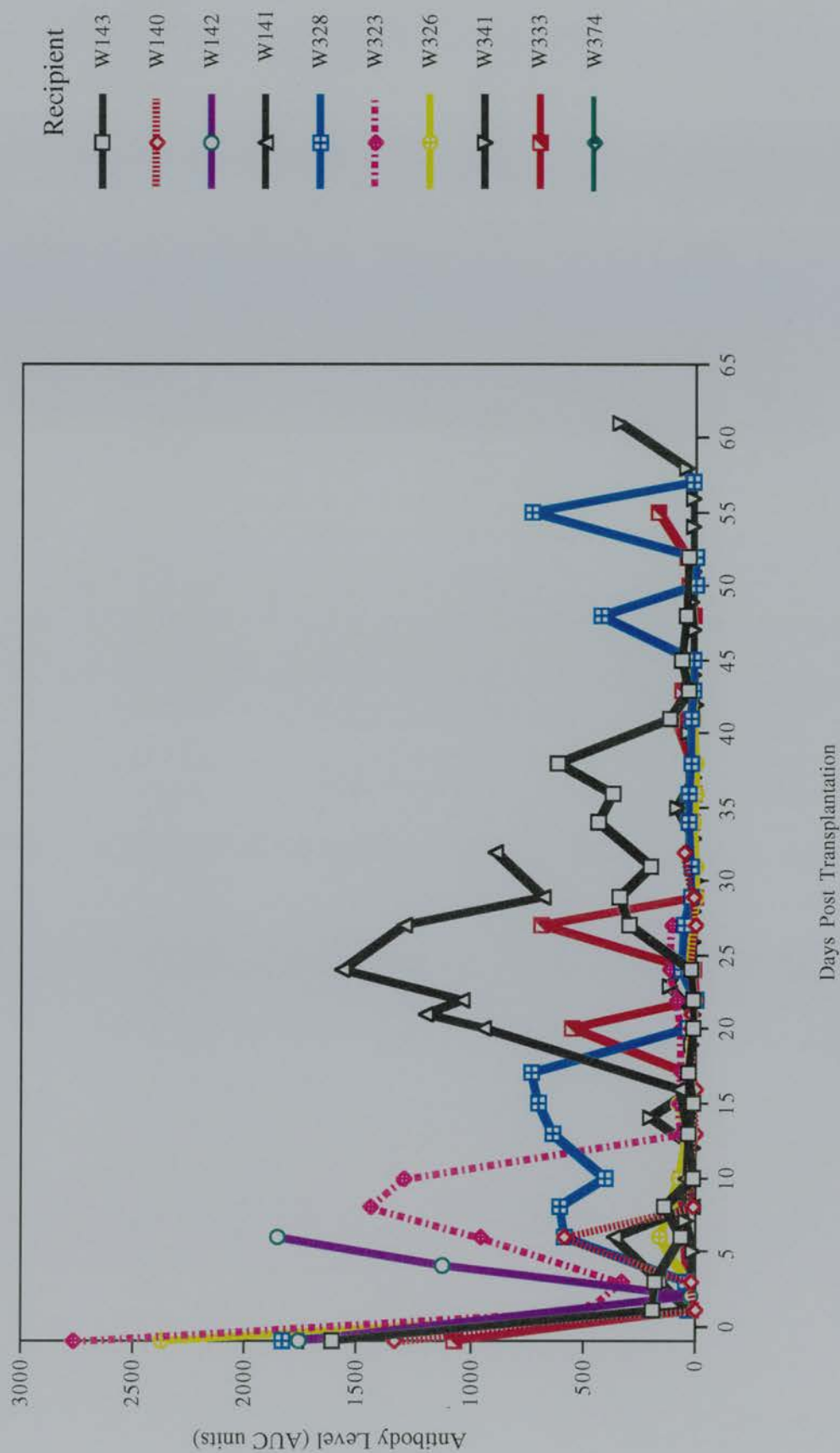


Figure 3.9. Total anti-pig antibody levels in the high cyclophosphamide group.

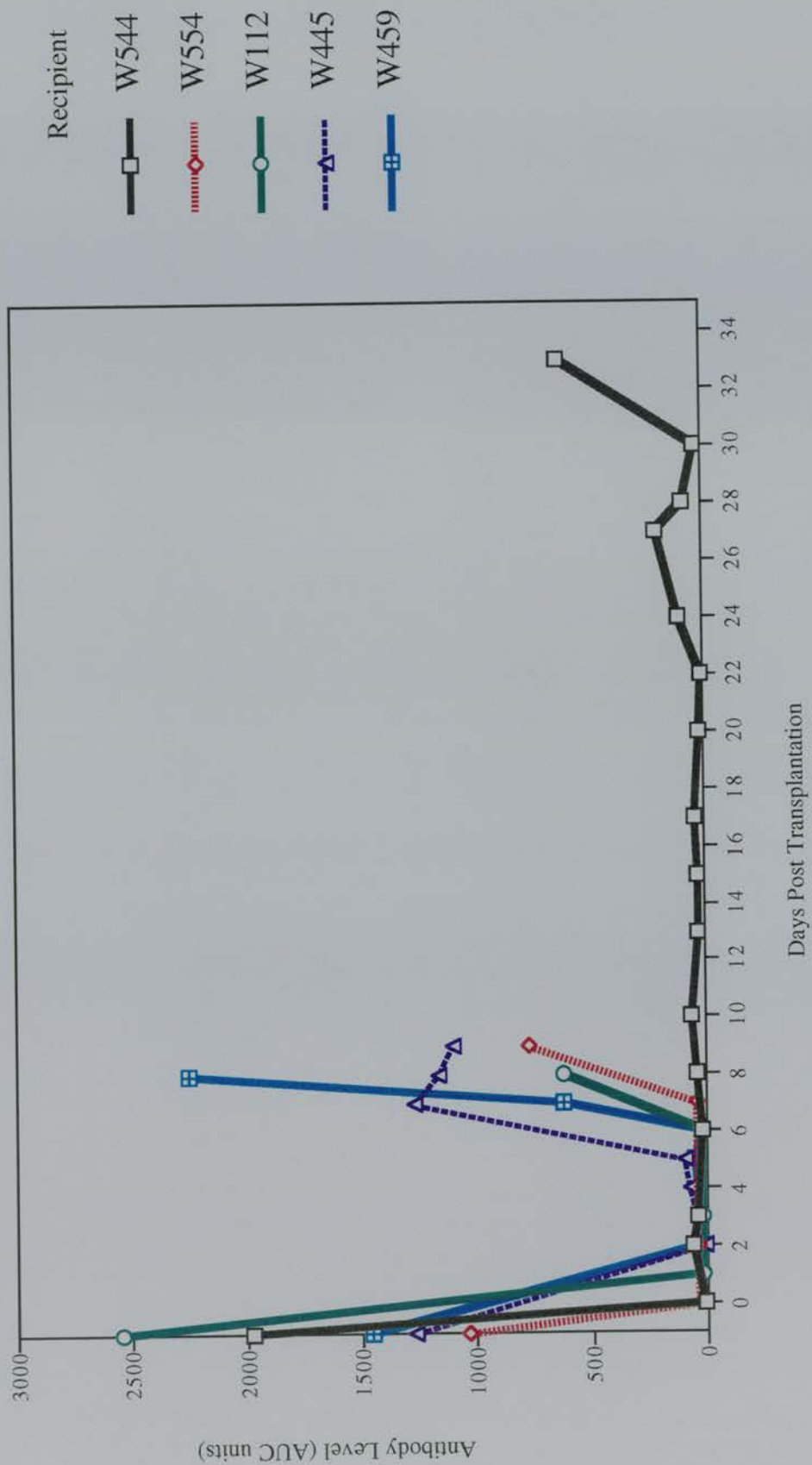


Figure 3.10. Total anti-pig antibody levels in the low cyclophosphamide group.

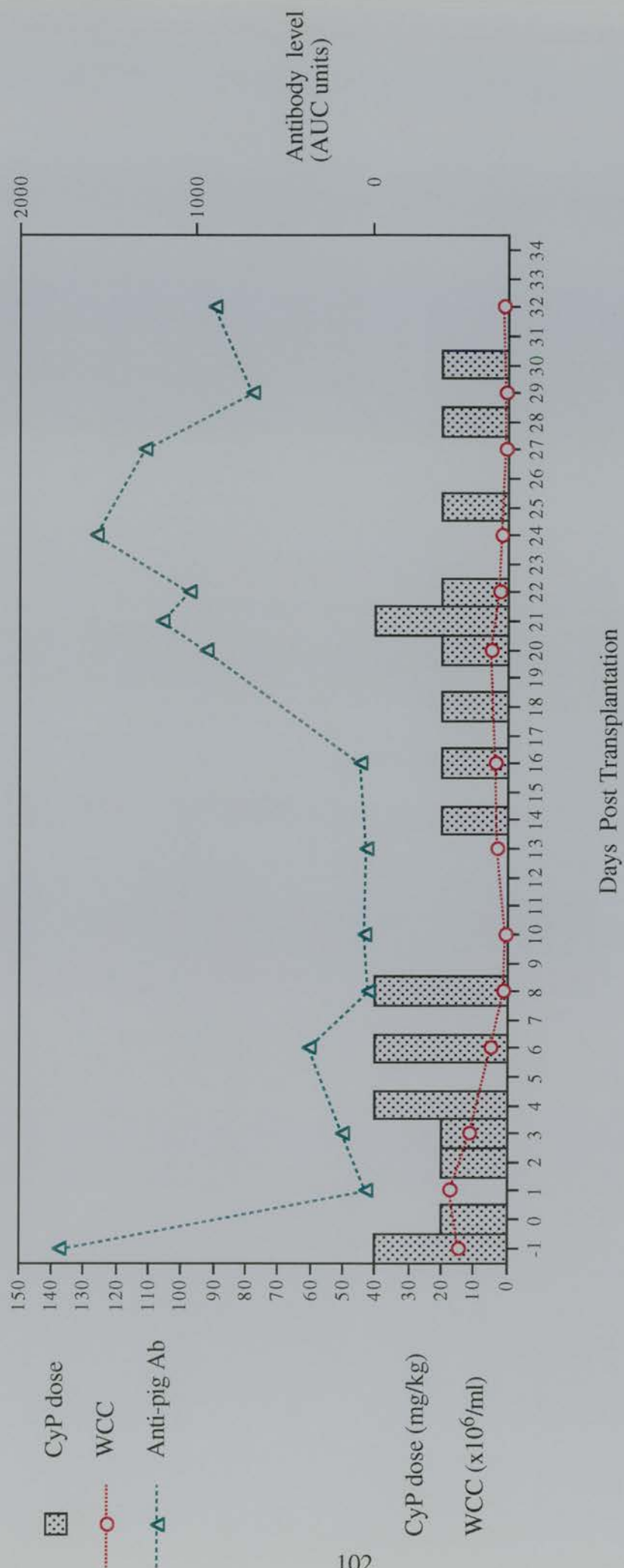


Figure 3.1.1. Variation of antibody level, white cell count and cyclophosphamide dose with time for W141 (Group 1).

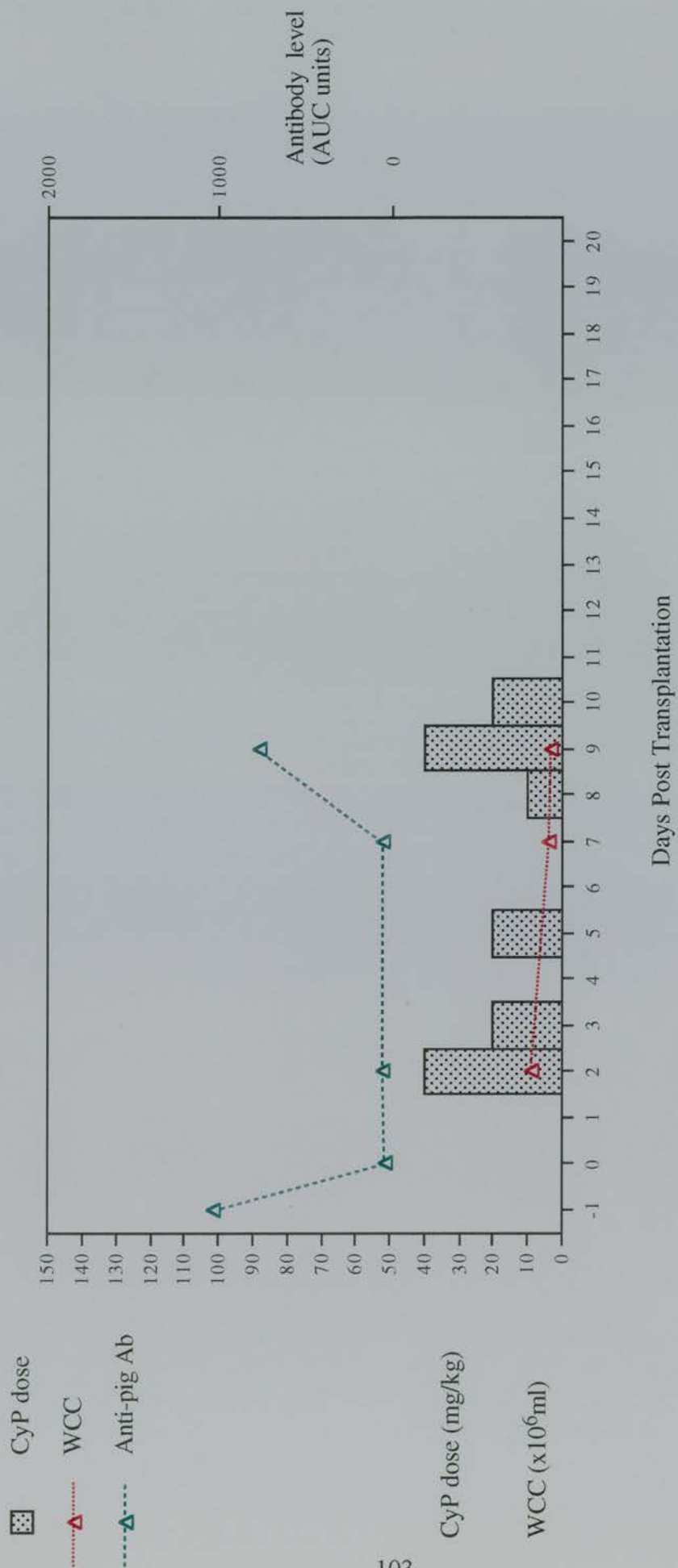


Figure 3.12. Variation of antibody level, white cell count and cyclophosphamide dose with time for W554 (Group 2).

Histology

All transgenic donor hearts scored 4 (scale of 0-4) for hDAF expression except that heart which was transplanted into recipient W142. At the time of explant of this pig heart it was found to express only 1+ of hDAF on its endothelium.

Histological examination of postmortem cardiac xenografts demonstrated florid HAR in all five control hearts (figure 3.13). There was no evidence of HAR in any of the 15 transgenic hearts. Two of ten transgenic hearts in group 1 (high CyP) were lost due to AVR: the remaining eight hearts in this group showed no evidence of rejection (figure 3.13). Of these eight hearts one failed due to a dilated cardiomyopathy and one heart stopped beating on day 55, but there was no evidence of rejection and no cause for its failure identified. The other six animals were sacrificed with beating cardiac xenografts in compliance with Home Office Animal Welfare guidelines as a result of gastrointestinal complications. All hDAF transgenic hearts in group 2 (low CyP) underwent AVR resulting in graft loss (figure 3.13).

The results of the grading for the histological parameters, immunohistological parameters and cell counts for immunosuppressed cynomolgus monkeys can be seen in tables 3.4. and 3.5.

Histology of group 1 hearts compared with controls

The median score for all histological parameters for group 1 xenografts was 0, there being no evidence of inflammation, cell damage or infiltrate. This contrasted markedly with the controls which had a median score of 4 for all but two histological parameters i.e. diffuse necrosis for which the controls had a median score of 1 and oedema for which the controls had a median score of 2.5. The only parameter for which this difference did not reach statistical significance was diffuse necrosis. The difference for congestion, focal necrosis, perivascular infiltrate, focal infiltrate, focal haemorrhage and oedema was significant at the level $p < 0.05$. The difference for white blood cell

adherence, platelet thrombi, diffuse infiltrate and aggressive infiltrate was significant at the level $p < 0.01$. The difference for retracted endothelial cells, endothelial permeability, endothelial rupture, vasculitis, thrombosis, fibrin, inflammatory cells, myocyte damage, myocytolysis, diffuse haemorrhage and endocardial white blood cell adherence was significant at the level $p < 0.005$. The difference for endocardial permeability and endocardial disruption was significant at the level $p < 0.001$.

Immunohistology of group 1 hearts compared with controls

The median score in the control xenografts was 4 compared with a median score of 2 or lower for each of the following parameters: C3 (figure 3.14), C9 (figure 3.15), P selectin and Von Willebrand's Factor (VWF). This was statistically significant at the level $p < 0.005$.

The median score in both the controls and the group 1 xenografts was 3 for C4. The median score for IgG deposition was 3 in group 1 and 4 in the controls, there being no significant difference. The median score for biotinylated griffonia simplicifolia lectin 1 (GSL.1), staining for gal α gal residues, was 3 in group 1 and 2 in the control xenografts, there being no significant difference.

There was no significant difference between the two groups for either the mean number of tissue platelets or tissue neutrophils per high power field.

Histology of group 2 hearts compared with controls

The difference between group 2 and the control xenografts for each of the following parameters was significant at the level $p < 0.05$: retracted endothelial cells, white blood cell adherence, endothelial permeability, thrombosis, platelet thrombi, fibrin, inflammatory cells, myocytolysis and focal necrosis. The median score for each of these parameters for group 2 xenografts was 2 to 2.5 compared with a median score for control xenografts of 4.

Again, there was a significant difference between group 2 and the control xenografts for each of the following parameters at the level $p < 0.01$: endothelial rupture, with median scores of 1 vs 4, endocardial white cell adherence (2 vs 4), endocardial permeability (2 vs 4) and endocardial disruption (0 vs 4).

Finally, there was no significant difference between the two groups (group 2 xenografts having a median score of 3 to 4 and control xenografts a median score of 4) for each of the following histological parameters: congestion, myocyte damage, perivascular infiltrate, focal infiltrate, diffuse infiltrate and aggressive infiltrate. There was no significant difference between the two groups for oedema, the median score being 3 for group 2 and 2.5 for control xenografts, or diffuse necrosis, the median score being 1 for both group 2 and control animals.

Immunohistology of group 2 hearts compared with controls

For each of the following immunohistological parameters there was a significant difference between group 2 and the control xenografts at the level $p < 0.05$: C3 (figure 3.14), C9 (figure 3.15), VWF and GSI.1 The median score for each parameter was 3 in group 2 and 4 in the control group. There was significantly more GSI.1 staining ($p < 0.05$) in group 2 than in the controls (3 vs 2). There was also a significant difference between group 2 and the control xenografts at the level $p < 0.005$ for P-selectin only, the median score for group 2 being 1 and the control xenografts being 4. There was no significant difference between the two groups for either C4, or IgG: the median score being 3 or 4 in each and finally, there was no difference in the mean number of tissue platelets or the mean number of tissue neutrophils per high power field in the two groups.

Histology of group 1 hearts compared with group 2 hearts

All histological parameters for group 1 transgenic xenografts had a median score of 0, there being no evidence of inflammation, cell damage or infiltrate for eight of the ten cardiac xenografts. The remaining two demonstrated evidence of AVR on days 6 and 62. Similarly, group 2 xenografts had median scores of 0 to 1 for diffuse necrosis, endothelial rupture and endocardial disruption, there being no significant difference between the two groups for these parameters. The median score in group 2 transgenic xenografts was 2 to 2.5, but not statistically different compared with group 1 xenografts for each of the following parameters: retracted endothelial cells, white blood cell adherence, endothelial permeability, vasculitis, myocytolysis and endocardial adherence. In group 2 transgenic xenografts the median score was 3 to 4 and again this was not statistically different compared with group 1 xenografts for each of the following parameters: platelet thrombi, myocyte damage, focal necrosis, perivascular infiltrate and focal haemorrhage.

However, the median in group 2 transgenic xenografts was 2 to 2.5 and this difference was statistically significant, compared with group 1 xenografts, at the level $p < 0.05$ for each of the following parameters: aggressive infiltrate, diffuse haemorrhage and endocardial permeability. For each of the following parameters: congestion, thrombosis, fibrin, inflammatory cells and focal infiltrate the median score in group 2 transgenic xenografts was 3 to 4 and was statistically different at the level $p < 0.05$. The median score in group 2 transgenic xenografts for oedema was 3 vs 0 for group 1 xenografts and this was also significant at the level $p < 0.05$.

Finally, the median score in group 2 transgenic xenografts for diffuse infiltrate was 4 vs 0 for group 1 xenografts and this reached statistical significance at the level $p < 0.01$.

Immunohistology of group 1 hearts compared with group 2 hearts

There was a significant difference ($p<0.05$) between group 1 and group 2 xenografts for VWF staining (2 vs 3) and a difference ($p<0.01$) for C3 (1 vs 3) and C9 deposition (0.5 vs 3). There was no difference between the two groups for either C4, IgG, or GSL.1 staining, both groups having a median score of 3 for each parameter. Nor was there a significant difference between the two groups for P selectin, the mean number of tissue platelets or the mean number of tissue neutrophils. A significant difference was found between group 1 and group 2 xenografts at the level $p<0.05$ for the mean number of CD-4 T cells, being $1\pm2.4/\text{HPF}$ in group 1 and $7\pm3.6/\text{HPF}$ in group 2 and the mean number of CD-8 T cells, being $8\pm8.9/\text{HPF}$ in group 1 and $23\pm10.8/\text{HPF}$ in group 2. There was no significant difference in the number of CD-19 B cells in each group.

It should be noted, however, that the sample numbers in these groups are small and that the mean cell counts for group 1 have large standard deviations indicating a large spread of results about the mean in a group which does not appear to contain completely parametric data (in this group two grafts were rejected and eight were not). Any conclusions about the mean cell counts in group 1 should therefore be treated with caution.

Histology of bone marrow

Histological examination of bone marrow of recipients of xenografts in both groups demonstrated no evidence of marrow suppression with a normal number of leukoerythroblastic precursors (figure 3.16).

Histology of bowel

Histological examination of sections of small and large bowel from those animals in group 1 which were sacrificed due to intractable diarrhoea demonstrated a non-specific ileitis/colitis consistent with CyP toxicity (figure 3.17).

Histological Parameter	Median Score			Statistical Significance		
	Group 1	Group 2	Controls	Group 1 vs Controls	Group 2 vs Controls	Group 1 vs Group 2
Retracted Endothelial Cells	0	2.5	4	p<0.005	p<0.05	NS
White Blood Cell Adherence	0	2	4	p<0.01	p<0.05	NS
Endothelial Permeability	0	2.5	4	p<0.005	p<0.05	NS
Endothelial Rupture	0	1	4	p<0.005	p<0.01	NS
Vasculitis	0	2	4	p<0.005	NS	NS
Congestion	0	4	4	p<0.05	NS	p<0.05
Thrombosis	0	3	4	p<0.005	p<0.05	p<0.05
Platelet Thrombi	0	3	4	p<0.01	p<0.05	NS
Fibrin	0	3	4	p<0.005	p<0.05	p<0.05
Inflammatory Cells	0	3	4	p<0.005	p<0.05	p<0.05
Myocyte Damage	0	3	4	p<0.005	p<0.05	NS
Myocytolysis	0	2	4	p<0.005	p<0.05	NS
Focal Necrosis	0	3	4	p<0.05	p<0.05	NS
Diffuse Necrosis	0	1	1	NS	NS	NS
Perivascular Infiltrate	0	4	4	p<0.05	NS	NS
Focal Infiltrate	0	4	4	p<0.05	NS	p<0.05
Diffuse Infiltrate	0	4	4	p<0.01	NS	p<0.01
Aggressive Infiltrate	0	2	4	p<0.01	NS	p<0.05
Focal Haemorrhage	0	3	4	p<0.05	NS	NS
Diffuse Haemorrhage	0	2	4	p<0.005	NS	p<0.05
Oedema	0	3	2.5	p<0.05	NS	p<0.05
Endocardial Adherence	0	2	4	p<0.005	p<0.01	NS
Endocardial Permeability	0	2	4	p<0.001	p<0.01	p<0.05
Endocardial Disruption	0	0	4	p<0.001	p<0.01	NS

Table 3.4. Grading and statistical significance of histological parameters for immunosuppressed cynomolgus monkeys.

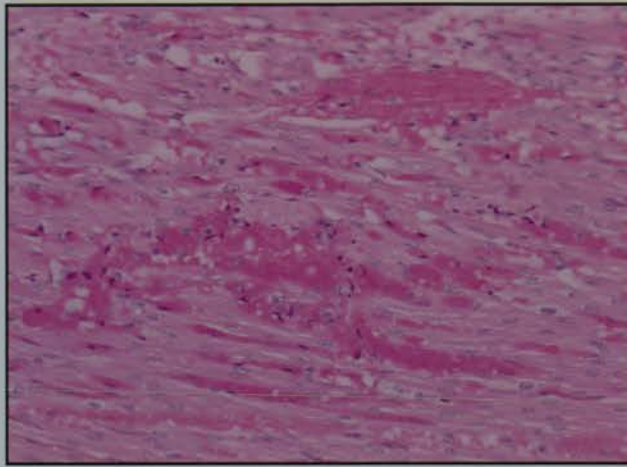
NS = not significant.

Note the median score was zero for all parameters in the group 1 (high cyclophosphamide) xenografts and that there was significantly more damage in group 2 (low cyclophosphamide) xenografts and even more damage in control xenografts for most parameters.

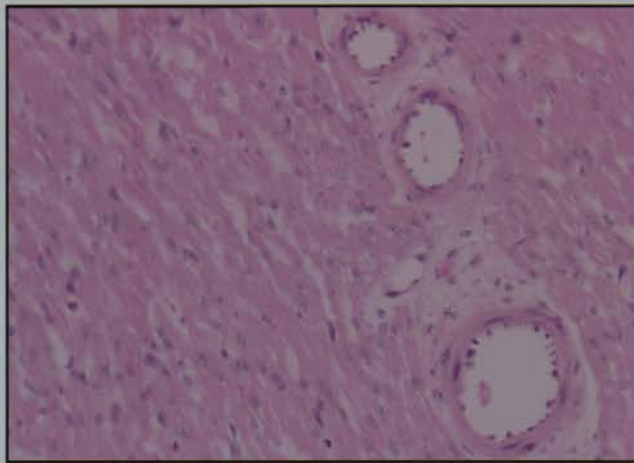
Immunohistological Parameters	Group 1	Median Score Group 2	Controls	Group 1 vs Controls	Statistical Significance Group 2 vs Controls	Group 1 vs Group 2
C3	1	3	4	p<0.005	p<0.05	p<0.01
C4	3	3	3	NS	NS	NS
C9	0.5	3	4	p<0.005	p<0.05	p<0.01
IgG	3	3	4	NS	NS	NS
P selectin	0	1	4	p<0.005	p<0.005	NS
Von Willebrand's Factor	2	3	4	p<0.005	p<0.05	p<0.05
GSL-1	3	3	2	NS	p<0.05	NS
		Mean Number/HPF				
Tissue Platelets	8	14	22	NS	NS	NS
Tissue Neutrophils	11	7	13	NS	NS	NS
CD-4 T Cells	1	7	-	-	-	p<0.05
CD-8 T Cells	8	23	-	-	-	p<0.05
CD-19 B Cells	0	8	-	-	-	NS

Table 3.5. Grading and statistical significance of immunohistological parameters for immunosuppressed cynomolgus monkeys.
NS = not significant, HPF = high power field.

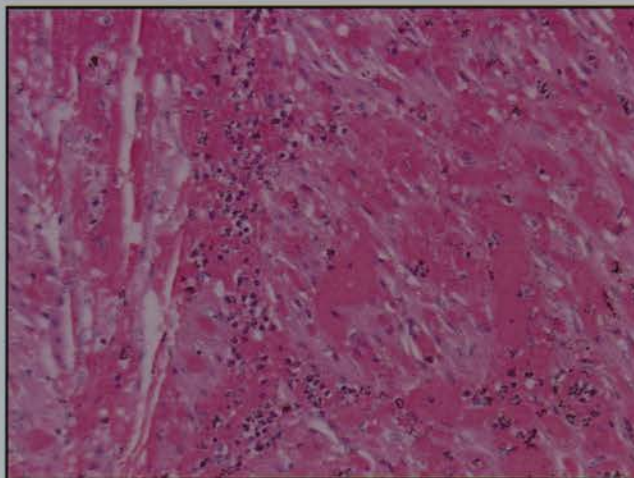
Note the difference in C3, C9, P selectin and Von Willebrand's Factor in the transgenic grafts and controls. Also note the significantly increased T cell infiltrate in group 2 (low cyclophosphamide) xenografts compared with group 1 (high cyclophosphamide) xenografts.



Control heart (35 mins) showing typical features of hyperacute rejection - thrombosis, haemorrhage, myocyte damage and neutrophil infiltration (x10).

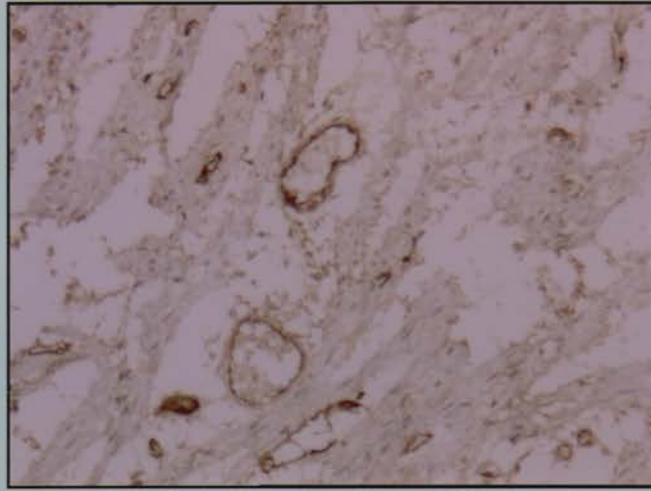


Group 1 hDAF transgenic heart (45 days) showing no rejection, with preservation of myocytes, no haemorrhage or thrombosis and clear blood vessels (x10).

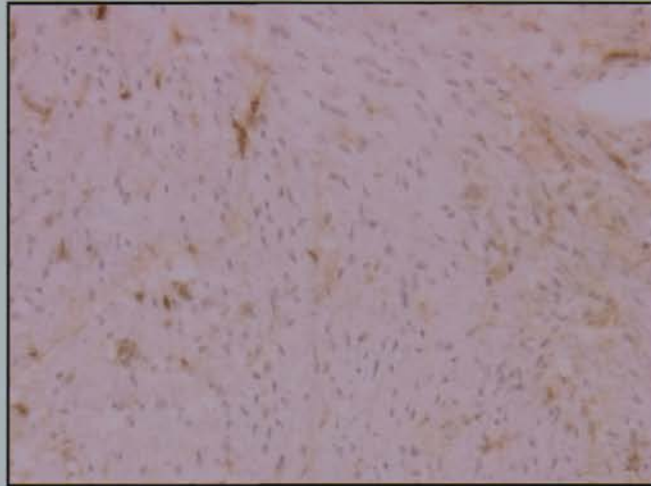


Group 2 hDAF transgenic heart (8 days) showing severe acute vascular rejection, with gross disruption of myocardial architecture, severe haemorrhage, and thrombosis with neutrophil infiltration (x10).

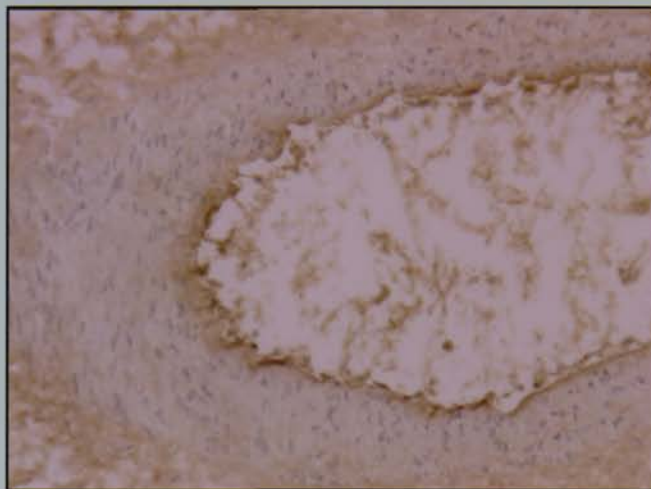
Figure 3.13. Sections of cardiac tissue, stained with haematoxylin and eosin, from heterotopically transplanted pig hearts, immunosuppressed with the group 1 (high cyclophosphamide) or group 2 (low cyclophosphamide) regimen.



Control heart (35 mins) showing heavy C3 deposition (x10).

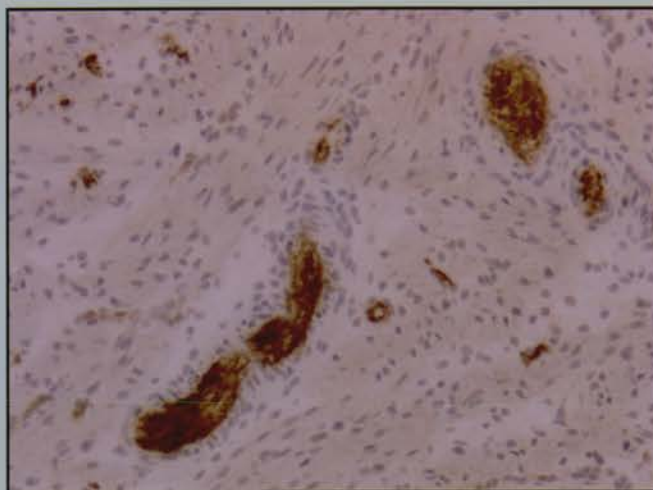


Group 1 hDAF transgenic heart (45 days) showing moderate C3 deposition (x10).

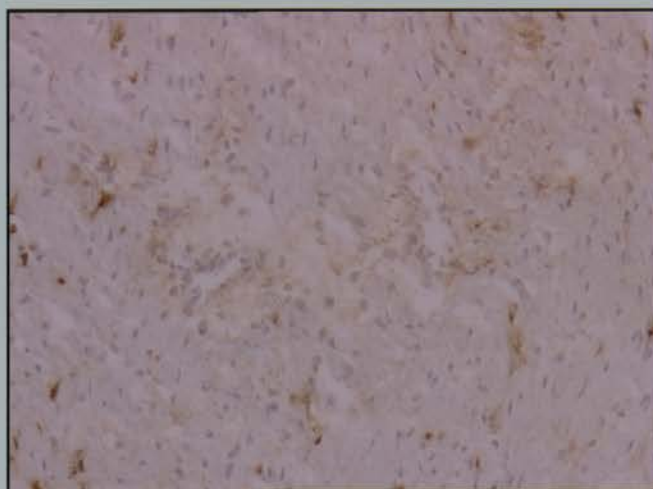


Group 2 hDAF transgenic heart (8 days) showing heavy C3 deposition on the endothelium of a large blood vessel (x20).

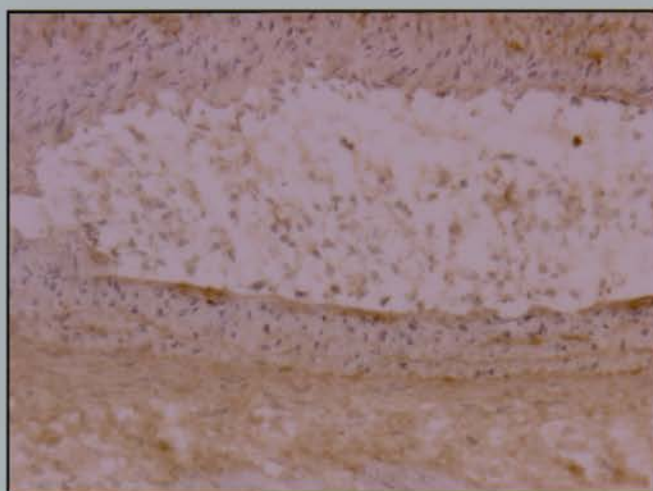
Figure 3.14. Sections of cardiac tissue, stained for complement component C3, from heterotopically transplanted pig hearts, immunosuppressed with the group 1 (high cyclophosphamide) or group 2 (low cyclophosphamide) regimen.



Control heart (35 mins) showing heavy C9 deposition (x10).



Group 1 hDAF transgenic heart (45 days) showing moderate C9 deposition (x10).



Group 2 hDAF transgenic heart (8 days) showing heavy C9 deposition (x20).

Figure 3.15. Sections of cardiac tissue, stained for complement component C9, from heterotopically transplanted pig hearts, immunosuppressed with the group 1 (high cyclophosphamide) or group 2 (low cyclophosphamide) regimen.

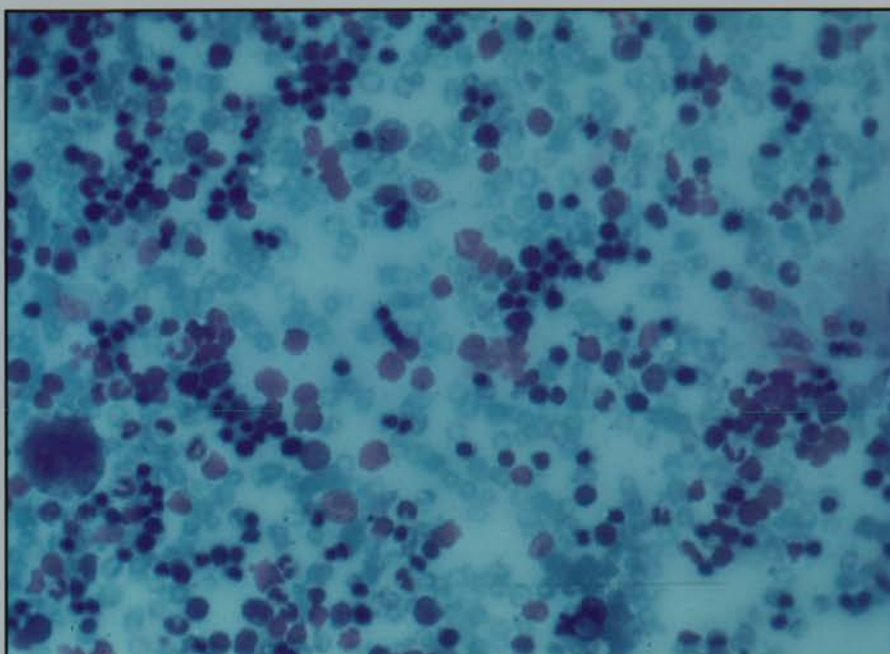


Figure 3.16. Histology photograph showing bone marrow stained with Giemsa from a group 1 (high cyclophosphamide) cynomolgus monkey. Note the normal leukoerythroblastic precursors in this immunosuppressed recipient (x10).

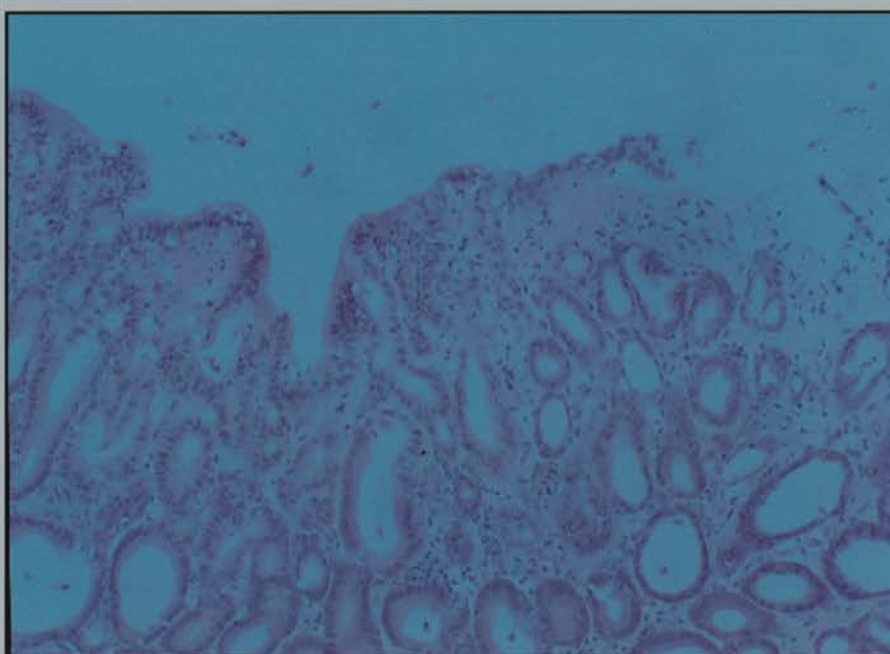


Figure 3.17. Histology photograph showing a section of bowel stained with haematoxylin and eosin from a group 1 (high cyclophosphamide) cynomolgus monkey which was sacrificed because of the development of intractable diarrhoea. Note the inflammatory infiltration and disruption of the epithelium consistent with cyclophosphamide toxicity (x10).

Discussion

We have already shown in chapter two that hDAF confers protection against HAR when hDAF transgenic pig hearts are transplanted heterotopically into the abdomen of non-immunosuppressed cynomolgus monkeys and that heterozygous hDAF transgenic pig hearts had a median beating time of 5.1 days. The aim of this part of the study was to determine the effect of a simple immunosuppressive regimen on prolongation of survival of heterozygous hDAF transgenic pig hearts in cynomolgus monkeys. CyP was chosen as the anti-B cell agent as prolonged survival of hamster-to-rat heart xenografts has been demonstrated when CyP was combined with CyA.⁹⁸

We have confirmed in this study that HAR can be abrogated with the hDAF transgene in this discordant pig-to-primate model. Indeed of the fifteen transgenic pig heart transplants performed none were hyperacutely rejected, whereas all five normal pig hearts were rejected hyperacutely.

It has also been demonstrated that the high dose, group 1, immunosuppressive regimen prolonged survival of xenografts out to 62 days (median survival 40 days). This contrasted markedly with the control hearts, all of which were hyperacutely rejected with a median beating time of 55 minutes. Only two hearts in the high CyP group were rejected, on days 6 and 62: all other hearts showed no evidence of rejection. Both rejected hearts demonstrated AVR histologically, but the heart which was rejected on day 6 was shown histologically to express very little hDAF on its endothelium (1+). This was also the first cynomolgus recipient to be immunosuppressed and it was clear that cynomolgus monkeys do not absorb CyA and CyP with the same profile as humans. As a result inadequate doses of drugs were administered to this recipient and the heart was rejected on day 6. Despite the apparent efficacy of the group 1 regimen 40% of recipients were sacrificed with beating cardiac xenografts due to intractable diarrhoea. This was thought to be a direct result of CyP toxicity in cynomolgus monkeys. In an attempt to reduce the dose of CyP administered

to the monkeys, and therefore the toxic side effects, and armed with in vitro evidence suggesting that CyP may be more effective if given shortly after the immunological stimulus,⁸⁷ no perioperative CyP was given to the group 2 monkeys. This resulted in a 44% reduction in the amount of CyP administered during the first nine days. None of the recipients developed diarrhoea but all five cardiac xenografts underwent AVR with a median survival of nine days.

Although the aim was to achieve a trough CyA level of 400ng/ml in both groups, and although the mean CyA dose administered to each group was similar, the mean CyA level achieved in the group 2 recipients was 876 ± 291 ng/ml compared with 554 ± 70.7 ng/ml in group 1. This difference didn't quite reach statistical significance ($p=0.07$). This observation may be accounted for by the troublesome diarrhoea in group 1 recipients perhaps interfering with CyA absorption and hence resulting in lower blood levels of the drug for a given dose. Interestingly, despite the higher CyA levels in group 2, it was the group 2 xenografts which were all rejected.

The immunosuppressive protocols chosen here could be applied clinically. The doses of CyP and cyclosporin given to the monkeys when measured in mg/kg were certainly large by clinical standards.⁸⁷ However these doses had to be increased to these levels to allow for the differences in absorption and metabolism of these drugs in cynomolgus monkeys, so that drug levels and haematological responses similar to those in current clinical practise could be achieved.

CyA was administered at doses necessary to achieve blood trough levels of 400ng/ml. Abnormal renal function did not occur in any of these animals. CyP was administered at doses necessary to achieve a total white count of 2×10^6 /ml. This threshold represents the haematological criterion currently used in the treatment of autoimmune diseases and is achieved with daily doses of 2-4mg/kg in man.⁸⁷ However, our group 2 monkeys required three times this dose to reduce the wcc to a mean of 4.7×10^6 /ml in the first nine days following transplantation. Intestinal complications associated with

the group 1 regimen were responsible for the sacrifice of 50% of the immunosuppressed monkeys with heart xenografts still beating. No infective cause was isolated from stool cultures and histological examination demonstrated a non-specific ileitis/colitis consistent with CyP toxicity. Diarrhoea has been reported in 22% of patients treated with CyP.⁸⁹ Clearly in the clinical setting appropriate supportive treatment would have been available.

Despite the reduction of the CyP dose administered to group 2 recipients the rate of fall of all haematological parameters i.e. total wcc, lymphocyte count, neutrophil count, haemoglobin concentration and platelet count was similar in both groups. The only difference was that the fall in group 2 lagged behind the fall in group 1 as might be expected when no perioperative CyP was given to group 2 recipients.

In all three groups the pretransplant natural APAb level in the cynomolgus monkey started at a level which was in excess of a pool of ten human sera, which was arbitrarily given a level (calculated from the area under the curve {AUC}) of 1000 units. Immediately following transplantation the APAb fell to almost zero, in both groups 1 and 2, presumably due to immunoabsorption onto the xenograft.

In group 1 the elicited total APAb levels, in general, remained low until about the third postoperative day, at which time they began to rise and peak by the fifth postoperative day. Beyond the fifth postoperative day there were a variable number of peaks and troughs of antibody levels. The amplitude of these peaks also varied widely. Despite the many antibody peaks, however, only two resulted in xenograft rejection.

In contrast, the pattern of elicited total APAb levels and their effect on graft viability in group 2 was markedly different. The levels initially remained low until about the fifth to eighth postoperative days, at which time there was again an increase in antibody levels in four out of five recipients, resulting in every case in xenograft rejection. The only recipient in group 2 in which there wasn't an antibody increase around this time was W544. In W544 the elicited total APAb level remained low until the 24th

postoperative day at which time there was a small peak and a later increase in antibody level on the 33rd postoperative day which resulted in xenograft rejection.

It appears that perioperative CyP in some way protected the grafts from later antibody attack, or that there may be more than one type of APAb involved, such that recipients in group 1 were able to tolerate such peaks of antibody whereas group 2 recipients were not.

We have confirmed in this study that HAR can be abrogated with the hDAF transgene in this discordant pig-to-primate model. Indeed, of the fifteen transgenic pig heart transplants performed, none were hyperacutely rejected whereas all five normal pig hearts were rejected hyperacutely. Two hearts in group 1 were rejected: all other hearts showed no evidence of rejection. Both rejected hearts demonstrated AVR histologically. All five group 2 cardiac xenografts underwent AVR.

A comparison of group 1 transgenic xenograft and control histology demonstrated no evidence of inflammation, cell damage or infiltrate in the eight transgenic grafts which were not rejected and while the control grafts were severely damaged. Those histological parameters for which there was a highly significant difference at the level $p < 0.001$ were endocardial permeability and endocardial disruption. This suggests that in this model, it was at the blood/endocardial interface where complement activation and therefore hDAF had its greatest effect.

There was heavy IgG, and C4 deposition in both groups but significantly more ($p < 0.005$) C3 and C9 deposition in the control grafts. This suggests that although there was substantial antibody binding to the transgenic grafts the activation of the terminal complement components was inhibited in the transgenic grafts. There was also less activation of the coagulation cascade in the transgenic grafts, as indicated by less VWF deposition ($p < 0.005$) and less endothelial activation, revealed by staining for P selectin ($p < 0.005$).

A comparison of group 2 transgenic xenograft and control histology demonstrated a similar pattern to that observed between group 1 transgenics and the controls although the degree of graft damage and histological changes in the group 2 xenografts was florid (features of AVR) and so the statistical difference between group 2 and control xenografts was less marked. Therefore, the amount of cellular infiltrate, myocyte damage and congestion was the same in both group 2 transgenics and the controls. Thus although the presence of hDAF on the pig endothelium was sufficient to protect against HAR it was not able to protect against AVR in the presence of rising APAb levels.

Again, there was heavy IgG and C4 deposition in both groups but significantly more ($p<0.05$) C3 and C9 deposition in the control grafts. There was still, however, substantial amounts of C3 (3+) and C9 (3+) deposited in the group 2 transgenic grafts. This would again support the hypothesis that complement activation plays an important part in the pathogenesis of AVR. Again, there was less activation of the coagulation cascade in the transgenic grafts, as indicated by less VWF deposition ($p<0.05$). The difference between group 2 transgenics and controls was however, statistically most significant for P selectin ($p<0.005$) indicating again that the presence of hDAF protects the pig hearts against endothelial cell activation.

A comparison of group 1 and group 2 transgenic xenograft histology demonstrated significantly more haemorrhage, thrombosis, oedema and cellular infiltrate in the group 2 xenografts. There was significantly more activation of the complement and coagulation cascades and more CD-4 and CD-8 T cells in the group 2 xenografts.

In summary HAR can be abrogated with the hDAF transgene in this pig-to-primate model and prolonged survival can be achieved. Perioperative CyP would appear to be an essential component of this regimen, without which, graft loss due to antibody-mediated AVR is encountered. There is clearly a need for an effective anti-B cell agent, with fewer gastrointestinal and haematological side effects, which can specifically

target elicited anti-pig antibodies and so overcome the next hurdle on the way to clinical pig-to-human heart transplantation. A number of potential agents now exist and these are described in the following section.

Leflunomide, an isoxazole, interferes with lymphocyte interleukin-2 responsiveness and has recently been shown to be a potent immunosuppressive agent.^{106,107}

Brequinar sodium is an antipyrimidine compound which noncompetatively inhibits the activity of the enzyme, dihydro-orotate dehydrogenase. Inhibition of the de novo pyrimidine biosynthetic pathway results in the depletion of the nucleotide precursors (uridine and cytidine) necessary for DNA and RNA synthesis. The dependence of lymphocytes on the de novo pyrimidine pathway for synthesis of DNA and RNA results in a high level of sensitivity to the action of brequinar during the proliferative phases of an active immune response. In vitro and in vivo experimental studies have confirmed the potent immunosuppressive characteristics of brequinar.^{108,109}

Rapamycin, a potent immunosuppressive agent, has been demonstrated to have remarkable activity in inhibiting allograft rejection in animal models of transplantation. It belongs to the class of macrocyclic immunosuppressive drugs that are bioactive only when bound to immunophilins. CyA and FK506 are also included in this class, but rapamycin acts at a later stage in the T cell cycle progression by blocking cytokine-mediated signal transduction pathways.¹¹⁰ Deoxyspergualin is effective in prolonging the functional survival of allogeneic and xenogeneic grafts through ill-defined mechanisms affecting macrophage and T and B cell function.¹¹¹ Mycophenolate mofetil is a new selective immunosuppressant used for the prevention and treatment of acute renal rejection after transplantation.¹¹² In vivo it is de-esterified to mycophenolic acid, which is a potent and specific inhibitor of de novo purine synthesis and suppressor of both T and B cell proliferation. Inhibition of vascular rejection in cardiac xenografts of cynomolgus monkeys transplanted into baboons has been demonstrated.¹¹³

Finally, sustained suppression of xenoreactive natural antibodies with anti-IgM monoclonal antibody¹¹⁴ has been demonstrated and monoclonal antibodies against CD4 and CD8 T cells in mice has been shown to prolong rat heart xenograft survival.¹¹⁵

As illustrated, there is an extensive armamentarium of new immunosuppressive agents currently available. Therefore one would hope that it should be possible to improve on the results which we have achieved in this study with a simple regimen of CyA, CyP and steroids in combination with the hDAF transgenic pig heart.

Chapter Four

Life supporting pig-to-baboon cardiac xenotransplantation

Introduction

We have already demonstrated in chapter two that hearts from hDAF transgenic pigs were not hyperacutely rejected when transplanted heterotopically into the abdomen of cynomolgus monkeys (*Macaca fascicularis*). We have further demonstrated in chapter three that prolonged survival of transgenic pig hearts was achieved when recipient cynomolgus monkeys were immunosuppressed with Cyclosporin A (CyA), Cyclophosphamide (CyP) and methylprednisolone (MP). The aim of the following studies was to determine whether transgenic pig hearts were able to support the life of a non-human primate.

In the earlier studies described in chapters two and three we chose the cynomolgus monkey as the recipient of heterotopic hDAF transgenic pig hearts largely because of the plentiful supply. However, such captive bred animals were rarely available with a body weight of greater than five kilograms. Hence the cynomolgus monkey was an unsuitable recipient for orthotopic heart transplantation due to the technical difficulties associated with cardiopulmonary bypass in such small recipients. Therefore, it was necessary to choose a larger primate for further studies. Many of the larger primates are endangered species and it was deemed ethically unacceptable to use such animals for this purpose. Of the larger primates readily available in sufficient numbers the baboon was considered to be the most suitable recipient. However, it has been shown that hDAF has a variable ability to down regulate complement of non-human primates (Tucker A and White D: personal communication). Indeed, hDAF has been shown to down regulate human complement activity by 84% of total plasma complement activity, cynomolgus complement by 72%, baboon complement by 69% and rhesus complement by 28%. Clearly the rhesus monkey would not be a good choice of experimental recipient for hDAF transgenic organs. However, it was not clear whether the 3% difference between the cynomolgus monkey and the baboon would be sufficiently large enough to result in HAR in the pig-to-baboon model. It was therefore

considered necessary to test hDAF in this model by transplanting hDAF transgenic pig hearts heterotopically into the neck of the baboon prior to performing orthotopic pig-to-baboon heart transplantation.

It has already been shown in a heterotopic pig-to-baboon model that complement depletion with cobra venom factor results in the delay of hyperacute rejection and that such grafts survived for up to 68 hours in this model without any other form of immunosuppression.⁴⁸ With a combination of CyP, 15-deoxyspergualin, splenectomy, baboon specific polyclonal anti-lymphocyte globulin and plasmapheresis survival of heterotopic pig hearts in baboons of 17 days has been achieved.⁶³ Pig-to-baboon functioning renal xenograft survival of up to 22 days has been achieved with a similar immunosuppressive regimen.¹¹⁶

The combination of cobra venom factor, splenectomy, CyA, prednisolone, CyP and methotrexate has resulted in pig-to-baboon heterotopic cardiac xenograft survival of 25 days.¹⁰⁴

Prolonged survival of life-supporting orthotopic pig hearts in the baboon for up to 16 days has also been achieved¹¹⁷ by using a radical immunosuppressive approach based on pretransplant splenectomy, recipient immunoabsorption and nafamstat mesilate. In addition, CyA and deoxyspergualin were administered to these animals. Histological investigations performed at the end of the study showed that even such a complex immunosuppressive regimen could not protect the graft from marked immunological damage.¹¹⁸

Materials and Methods

Donor selection

Large white/Landrace cross-piglets of either sex were selected as donors on the basis of degree of hDAF expression, as described in chapter two, and on the basis of their body mass. All donors were heterozygous for hDAF.

Heterotopic cervical model (n=3)

2-3 kg piglets were used (i.e. as small as possible) because of the restricted space within the baboon neck.

Orthotopic model (n=5)

5-6 kg piglets were used as these were found to have comparable size hearts to the recipient baboons thus allowing anastomoses of vessels of similar sizes and allowing the heart to sit within the pericardial space without being tamponaded.

Recipient selection

For both the heterotopic and orthotopic models wild-caught baboons (*Papio anubis*, age 2-4 years, weight 6.8-9.3kg) as large as possible were used so that the technical difficulties associated with these procedures were minimised.

Donor operation

Heterotopic cervical cardiac transplantation

This was as described for heterotopic abdominal cardiac transplantation in chapter two.

Orthotopic cardiac transplantation

After sedation with ketamine hydrochloride (20mg/kg) and oxygen mask induction, a tracheostomy was performed and mechanical ventilation was begun. General anaesthesia was maintained with N₂O:O₂ (1:1) and isoflurane (1-2%).

A median sternotomy was performed, the pericardium was opened longitudinally, and heparin (300 iu/kg) was given intravenously. The inferior vena cava (IVC) was tied above the diaphragm, and incisions were made into the IVC and the left superior pulmonary vein to decompress the heart. The aorta was then cross-clamped at the level of the right brachiocephalic artery and St. Thomas's cardioplegic solution (15ml/kg) was infused at a temperature of 4°C into the aortic root. The heart was excised after division of the superior vena cava, IVC, pulmonary veins pulmonary artery and aorta. The heart was kept cool by topical irrigation with cold normal saline at 4°C. Simple defects such as a patent foramen ovale were repaired.

Recipient operation

Heterotopic cervical cardiac transplantation

All animals were starved overnight prior to surgery. Before transfer to the operating theatre the baboon was sedated with ketamine hydrochloride (15mg/kg) given intramuscularly in combination with atropine sulphate (0.05mg/kg).

Following induction of anaesthesia, by intravenous injection of diazepam (1mg/kg), an endotracheal tube was inserted and the baboon was maintained on a N₂O:O₂ (1:1) mixture, with the addition of isoflurane at a concentration of 1-2% as required and propofol (50mg/kg). A single dose of a prophylactic antibiotic was given (Synulox). Analgesia was achieved by intravenous fentanyl citrate. An indwelling cannula was inserted into a peripheral vein for administration of fluids and drugs during the procedure. Electrocardiographic and respiratory monitoring was established.

The skin over the right side of the baboon's neck was then prepared with antiseptic solution and sterile drapes were applied to expose the operative field. An oblique neck incision was performed which was taken down through platysma and a self-retaining retractor was inserted. A 2-3cm long segment of internal jugular vein and internal carotid artery was then exposed. Donor ascending aorta was anastomosed to the

recipient internal carotid artery and the donor pulmonary artery was anastomosed to the recipient internal jugular vein.¹¹⁹

100 iu of heparin was given intravenously and allowed to circulate for two minutes prior to occluding the internal jugular vein and internal carotid artery using silastic slings. With the heart wrapped in a cold saline-soaked swab, and kept cold at all times, the anastomoses were performed using 7-0 prolene sutures (non-absorbable monofilament) and standard microvascular surgical techniques. On completion of the anastomoses the vessels were flushed with heparinised saline and all air was expelled. After checking the anastomoses for any obvious surgical bleeding the slings were carefully removed (the ischaemic time noted) and the surgical field was gently packed with swabs. 20mls of haemaccel (plasma expander) was given intravenously. If the heart did not spontaneously cardiovert a 2J DC shock was administered and repeated if required. The swabs were then removed, the surgical field was carefully examined for bleeding and thorough haemostasis was achieved. Finally, thrombin glue (Tisseal glue, Immuno Ltd High Wycombe, UK) was applied around the anastomoses and the heart was carefully positioned to avoid compromising the activity of the donor heart. When we were satisfied that there was stable xenograft function and adequate haemostasis the wound was closed with 3-0 vicryl sutures (absorbable) to close the platysma and 3-0 subcuticular vicryl for the skin. The recipient was allowed to recover from general anaesthesia in the most appropriate position for the graft and was maintained in an incubator until sitting up, at which time it was returned to its cage.

Postoperative management

First hour

This period was critical. The animal was observed very closely during this period and kept in the operating theatre with full anaesthetic and surgical facilities available, should it have been necessary to reopen the cervical wound.

Hours 2-24

Postoperative analgesia (buprenorphine hydrochloride, 0.3mg/ml) was administered at the discretion of the attending veterinary surgeon.

During the first 24 hours the donor heart was checked every hour to confirm that it was beating and if there was any problem with it, or with the animal, the surgeon was notified.

Day 2 onwards

The general physical condition of the recipient and the xenograft function was checked three times each day and any problem was reported to the on-call surgeon.

Orthotopic cardiac transplantation

Preoperative venesection

Cardiopulmonary bypass in very small animals is technically difficult. Cardiopulmonary bypass circuit procedures can lead to marked haemodilution. Blood-based prime reduces such effects. Human-type ABO blood grouping can easily be performed in baboons, but without a readily available bank of typed baboon blood it is of limited use. Simian-type blood grouping is more complex.⁷⁰ In order to circumvent these difficulties each recipient baboon was venesected (9mls/kg) on two separate occasions each 28 days apart. The second venesection was 28 days prior to transplantation. The blood was frozen at -35°C and stored at the National Blood Service (Birmingham Centre, UK) and then defrosted on the day of transplantation. By retransfusing the stored blood it was possible to avoid significant anaemia following cardiopulmonary bypass.

Recipient operation

Through a median sternotomy the cardiectomy and orthotopic heart transplantation was performed using the atrial cuff technique.¹²⁰

Meticulous surgical technique and haemostasis were essential. After implantation and discontinuation of cardiopulmonary bypass the chest was closed leaving a single chest drain in the pericardium.

Postoperative management

First hour

If significant bleeding did not occur within one hour, the drain was removed and the animal was extubated, otherwise the chest was reopened immediately and the source of the bleeding was identified and rectified.

Hours 2-24

Having completed the operation the animal was allowed to recover in an incubator and subsequently transferred to an oxygen-enriched cage without intravenous fluid replacement or inotropic support. Postoperative analgesia (buprenorphine hydrochloride, 0.3mg/ml) was administered at the discretion of the attending veterinary surgeon.

During the first 24 hours the baboon was checked every hour and if there was any problem the surgeon was notified.

Day 2 onwards

The general physical condition of the baboon was checked three times each day and any problem reported to the on-call surgeon.

Immunosuppression

Cyclosporin A

All animals in both groups received CyA to achieve trough levels in excess of 600 ng/ml. A target cyclosporin level as high as 1500ng/ml has been recommended to have comparable effects on baboon lymphocytes when compared with human lymphocytes.¹²¹

Steroids

MP was commenced at 1mg/kg/day reducing to 0.2mg/kg/day by day 18.

Cyclophosphamide

CyP was given intravenously on the preoperative day at 40mg/kg, the operative day at 20mg/kg, the second postoperative day at 10-40mg/kg and alternate days thereafter, tailored to the white cell count (wcc) and the total haemolytic red blood cell anti-pig antibody (APAb) levels. This immunosuppressive regimen was similar to that received by the group 1, or high CyP, cynomolgus monkey recipients in chapter three. CyP was administered to achieve a minimum total wcc of $2 \times 10^6/\text{ml}$. This minimum wcc reflects experience which has recently been gained in clinical immunosuppression of patients suffering from autoimmune diseases such as systemic lupus erythematosus.⁸⁷ Some authors even recommend a wcc as low as $1.5 \times 10^6/\text{ml}$ as the minimum acceptable level for immunosuppression in patients with lupus nephritis.¹²² In two orthotopic baboons the postoperative CyP was reduced, each baboon receiving a total of 10mg/kg.

Blood sampling and monitoring

Blood was sampled regularly for full blood count and differential white cell count (Bayer-Technicon haematology analyser), electrolytes and creatinine (Hitachi 737 clinical chemistry analyser) trough CyA levels (thin-layer chromatography, Papworth Hospital), total red blood cell haemolytic anti-pig antibody (APAb) levels (as described in chapter 2) and the immunosuppression was tailored accordingly.

Diagnosis of Xenograft Rejection

Heterotopic cervical model

Rejection of the heterotopic hearts was defined as the absence of palpable cardiac pulsation confirmed by histological examination of the graft.

Orthotopic model

Rejection of the orthotopic grafts was diagnosed by a rise in the total haemolytic red blood cell APAb levels and/or by clinical signs of heart failure (weight increase, oedema, dyspnoea and cardiac dysrhythmia) and confirmed by histological examination of the grafts.

Histology

Donor

All donor ear clippings were scored for hDAF expression as described in chapter 2. Only top-scoring hDAF transgenic organs were used.

Recipient

All explanted hearts were examined histologically with haematoxylin and eosin (H+E) staining for each of the parameters as described in chapter two. All histological parameters were graded on a scale of 0 to 4.

Immunohistology

Cardiac tissue was also snap-frozen in liquid nitrogen and stained for each of the immunohistological parameters using mouse anti-human C3, C4, C9 and IgG monoclonal antibodies (mAbs)(Dako Ltd, High Wycombe, UK) and IgM (mouse anti-human mAb, Immunotech SA, Marseilles, France) by the avidin-biotin complex method. Explanted grafts were evaluated for galactose α 1-3 galactose expression. This was determined by measuring the intensity of biotinylated Griffonia (bandeiraea) simplicifolia Lectin 1 binding (GSL: Vector, California, USA). Explanted grafts were also stained for the adhesion molecule P selectin using a mouse anti-human mAb against P selectin (Immunotech, Hamburg, Germany) and Von Willebrand's Factor

(VWF) using mouse anti-human mAb against VWF (DAKO Ltd, High Wycombe, UK). These parameters were measured semi-quantitatively on a scale of 0 to 4.

The cellular infiltrate was evaluated in each graft by counting the mean number of cells per high power field (HPF). Tissue platelets were identified by mouse anti-human mAb against P selectin (Immunotech, Hamburg, Germany) and neutrophils by mouse anti-human neutrophil mAb. CD-4 T helper cells using mouse anti-human CD-4 mAb (DAKO Ltd, High Wycombe, UK), CD-8 T cytotoxic cells using mouse anti-human CD-8 mAb (DAKO Ltd, High Wycombe, UK), CD-19 B cells using mouse anti-human CD-19 mAb (DAKO Ltd, High Wycombe, UK) and CD-68 macrophages using mouse anti-human CD-68 mAb (DAKO Ltd, High Wycombe, UK).

Bone marrow examination

Bone marrow from selected animals was stained with Giemsa to demonstrate haematological precursors.

Results

The results are summarised in table 4.1.

Recipient	Survival	Outcome
Heterotopic 1	13 days	Acute vascular rejection
Heterotopic 2	2 days	Sacrificed : cerebrovascular accident
Heterotopic 3	21 days	Sacrificed: cervical abscess eroding internal jugular vein
Orthotopic 1	9 days	Bone marrow suppression
Orthotopic 2	5 days	Acute vascular rejection
Orthotopic 3	5 days	Acute vascular rejection
Orthotopic 4	11 hours	Technical failure: pulmonary artery thrombosis
Orthotopic 5	6 hours	Technical failure: left ventricular infarction

Table 4.1. Summary of hDAF transgenic pig-to-baboon cardiac xenotransplantation.

Heterotopic hearts (n=3) None of the transgenic pig hearts were hyperacutely rejected. One heart underwent AVR on day 13 in association with a rising total APAb (figure 4.3) and the remaining two recipients were sacrificed with beating xenografts, one on day two, following a cerebrovascular accident, and one on day 21 due to a cervical abscess eroding the internal jugular vein leading to haemorrhage and collapse of the animal. In the latter two recipients there was no evidence of rejection.

Armed with the knowledge that the hDAF transgene is clearly able to abrogate HAR in this pig-to-baboon heterotopic heart transplantation model we were able to proceed onto the second phase of this experiment i.e. life-supporting hDAF transgenic pig-to-baboon orthotopic heart transplantation.

Orthotopic hearts (n=5) None of the hearts were hyperacutely rejected. Two xenografts failed due to technical reasons, one failed 11 hours after transplantation due

to a kinked pulmonary artery anastomosis leading to right heart failure and thrombus formation in the right atrium and ventricle. A second graft failed six hours after transplantation due to dysrhythmia secondary to left ventricular infarction.

Two xenografts stopped beating on day five in the presence of rising total APAb (figure 4.3) and histological examination demonstrated AVR. Of these two recipients, the recipient of the third orthotopic baboon heart (orthotopic 3) underwent two consecutive orthotopic heart transplantations during the same operation. The first graft failed after 20 minutes and so a second hDAF transgenic pig heart was transplanted which survived for five days.

Finally, one recipient was sacrificed on day nine with a life-supporting xenograft. At the time of sacrifice this recipient was pancytopenic with a haemoglobin of 8.8g/dl, total white blood cell count of $0.15 \times 10^6/\text{ml}$ and a platelet count of zero. This haematological state lead to widespread petechial haemorrhage and poor general condition. Bone marrow examination confirmed marrow suppression.

Surgical details of orthotopic heart transplantation

Recipients of orthotopic hearts 1 and 2 survived the procedure without any lasting perioperative insult. However, the xenograft in orthotopic 3 failed after only 20 minutes without any obvious cause. The heart became engorged, mottled and purple. It then developed a bradycardia and stopped beating. Therefore, a second donor pig was rapidly prepared, the heart was harvested, and under the same anaesthetic, and with the recipient baboon maintained on cardiopulmonary bypass, a second orthotopic heart transplant was performed (hence the very long bypass time of 238 minutes for this recipient). This xenograft went on to function well.

Technical difficulties were experienced with orthotopic 4. At the time of the operation it was clear that there was a large donor-recipient organ size mismatch (+57.8% - see table 4.2).

Recip-ient	Survival	Outcome	CPBT (min)	IT (min)	Weight-mismatch (+%)	APAb (AUC)
1	9 days	Bone marrow suppression	59	40	55.3	1024
2	5 days	Acute vascular rejection	70	64	39.4	967
3*	5 days	Acute vascular rejection	238	53	30	1152/98
4	11 hours	Pulmonary artery thrombosis	110	54	57.8	919
5	6 hours	Primary graft failure	74	61	30.6	1133

3* = recipient received two transplants

CPBT = Cardiopulmonary bypass time, IT = Ischaemic time

APAb = Anti-pig antibody, AUC = Area under curve units

Table 4.2. Surgical details and preoperative antibody levels in orthotopic baboons.

This resulted in a very difficult pulmonary artery anastomosis and created a long pulmonary artery which may have led to it kinking. The degree of difficulty of this operation is also reflected in the relatively long cardiopulmonary bypass time of 110 minutes. This xenograft stopped beating 11 hours following transplantation and at the time of the postmortem the pulmonary artery was found to contain thrombus proximal to a kinked pulmonary artery. Histological examination demonstrated no evidence of rejection.

Although the operation on orthotopic 5 was uncomplicated, on removing the aortic cross clamp and allowing the xenograft to reperfuse, ventricular fibrillation and supraventricular tachycardia (SVT) were problematic. This graft later failed at six hours and histological examination suggested that this was due to ventricular fibrillation secondary to left ventricular infarction.

On completion of the anastomoses and reperfusion of the grafts five of the six grafts developed ventricular fibrillation requiring 2 Joules DC cardioversion. All were successfully converted to either sinus rhythm or SVT. Amiodarone (5mg/kg) was administered to treat SVT. Bradycardic episodes and low output states were treated with a combination of 1mmol boluses of calcium chloride and 0.1ml of 1:10,000 adrenaline. Prolonged inotropic administration was not utilised.

Immunosuppression

Cyclophosphamide

All heterotopic baboons received the same CyP regimen as group 1 cynomolgus recipients in chapter three i.e. the high CyP regimen. The first orthotopic baboon, however developed profound marrow suppression and so the CyP dose was reduced in the following two recipients from a mean daily dose of 15mg/kg to 10mg/kg. As with immunosuppressed cynomolgus recipients of pig hearts in chapter three the CyP dose was tailored to the wcc and the total haemolytic red blood cell APAb levels. CyP was administered to achieve a minimum total wcc of $2 \times 10^6/\text{ml}$. The mean daily dose of CyP administered in an attempt to achieve these goals in both heterotopic and orthotopic baboons was $11.1 \pm 2.2 \text{ mg/kg}$.

Cyclosporin A

An attempt was made to achieve trough levels in excess of 600ng/ml. The actual mean CyA level achieved in the heterotopic and orthotopic baboons was $987 \pm 76 \text{ ng/ml}$ during the experiment. This required a mean daily CyA dose of $177 \pm 13 \text{ mg/kg}$.

Electrolytes and creatinine levels

Electrolyte and creatinine levels were normal in all recipients. Postoperative creatinine levels ranged from 0.7-1.3mg/dl (normal range 0.5-1.5mg/dl).

Total white cell count

We can see from figure 4.1 that the mean total wcc in baboons following transplantation with hDAF transgenic pig hearts, when immunosuppressed with CyA, CyP and steroids, initially rose from a mean pretransplant level of $11.6 \pm 2.1 \times 10^6/\text{ml}$ (normal range $5.1\text{--}16.4 \times 10^6/\text{ml}$) to $12.5 \pm 1.2 \times 10^6/\text{ml}$ on the first postoperative day.

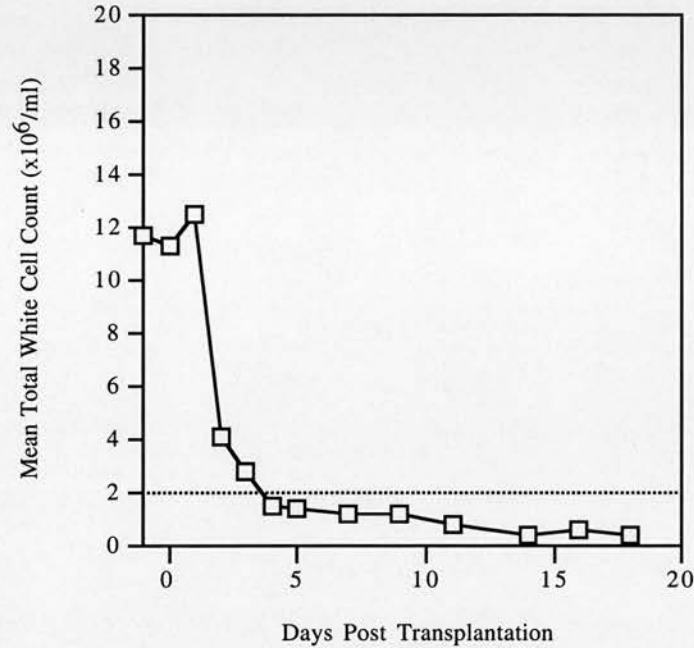


Figure 4.1. Mean total white cell count in baboons.

This was similar to the response seen in cynomolgus monkeys in chapter 3 and was likely to represent the inflammatory reaction to surgery and indeed was mirrored by a rise in neutrophils. Following this initial rise there was a rapid fall in the mean total wcc such that by the fourth postoperative day the mean total wcc had fallen below $2 \times 10^6/\text{ml}$. Beyond the fourth postoperative day, although there were fluctuations in the wcc, it continued to fall and despite simultaneous reductions in the CyP dose it didn't recover.

Lymphocyte count

The mean lymphocyte count in baboons, following transplantation with hDAF transgenic pig hearts, immunosuppressed with CyA, CyP and steroids can be seen in figure 4.2. We can see that there was initially a rapid fall from the pretransplant level of $5.6 \times 10^6/\text{ml}$ (normal range $2.48\text{--}11.24 \times 10^6/\text{ml}$) to a post-transplant level of $1 \times 10^6/\text{ml}$ on the first postoperative day.

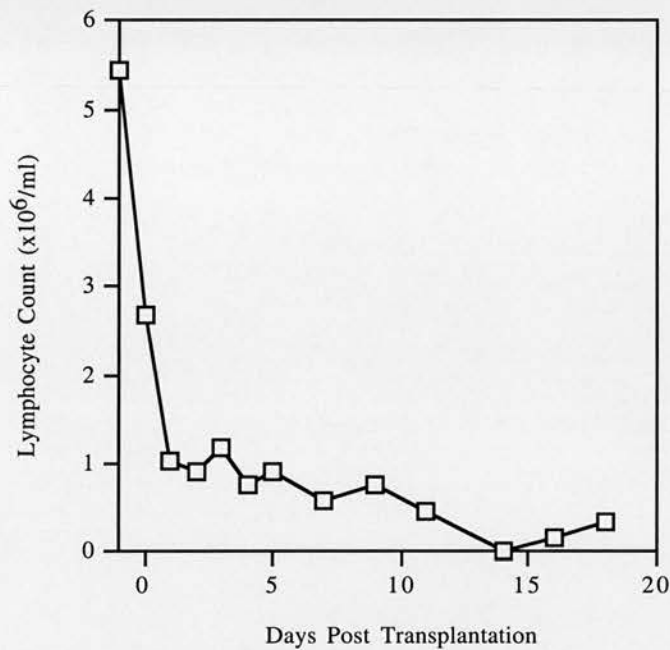


Figure 4.2. Mean lymphocyte count in baboons.

Thereafter there were fluctuations in the mean lymphocyte count but there was a clear downward trend until the fourteenth postoperative day, following which there was a slight recovery of the lymphocyte count which followed a reduction in the CyP dose.

Total anti-pig antibody levels

Figure 4.3 shows the pre and post-transplant total APAb levels in heterotopic and orthotopic baboons surviving beyond the first post-transplant day. We can see from figure 4.3 that the total APAb levels in both the heterotopic and orthotopic baboons initially started at pretransplant levels of between 430 and 1152 units and immediately following transplantation all fell to around zero (presumably due to immunoabsorption onto the graft). Note that in orthotopic 3 the pretransplant total APAb level started at 1152 units and that the first pig heart grafted failed with no obvious cause identified. During the same anaesthetic a second pig heart was transplanted successfully which went on to support the life of the recipient baboon for five days.

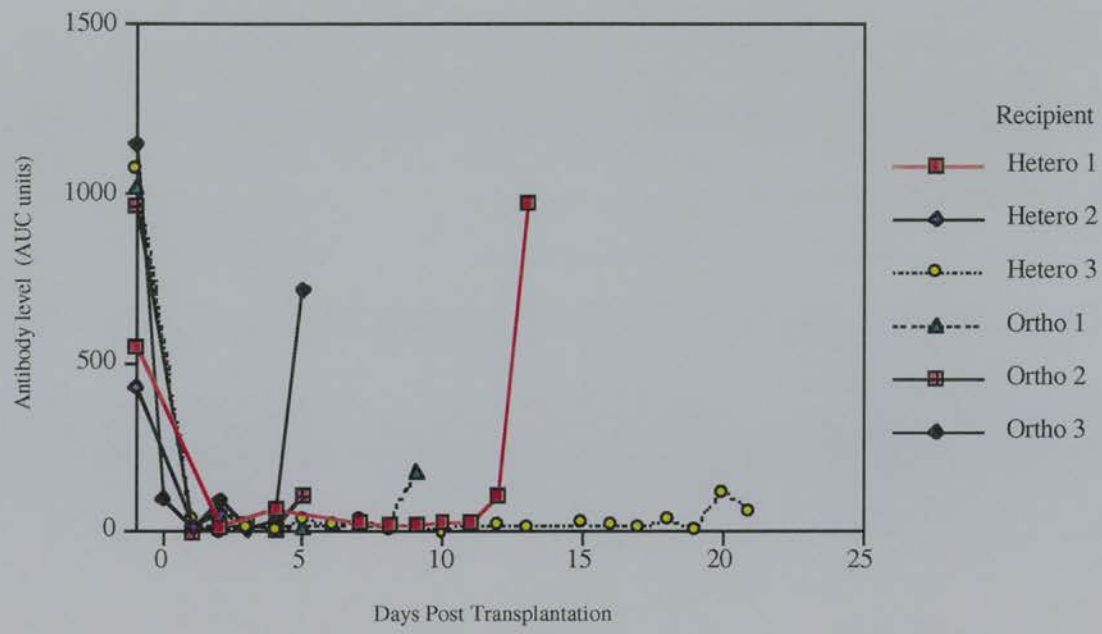


Figure 4.3. Total anti-pig antibody levels in heterotopic and orthotopic baboons.

Following the explant of the first transplanted heart, and prior to implantation of the second heart, the total APAb level was found to be 98 units and therefore the second graft may have been relatively protected against anti-pig antibodies by the initial

perfusion of baboon blood through the first pig heart, thus facilitating immunoabsorption of anti-pig antibodies onto the graft.

The other two orthotopic hearts which were thought to fail for technical reasons, i.e. orthotopics 4 and 5, had pretransplant total APAb levels of 919 units and 1133 units respectively (see table 4.2). So it would appear that there was no difference in the pre-transplant total APAb levels compared with those grafts that survived beyond the first postoperative day. Histological analysis of these two grafts demonstrated no evidence of rejection.

We can see from figure 4.3 that the elicited post-transplant total APAb level in heterotopic 1 remained suppressed until the 12th postoperative day when it suddenly started to climb and, despite an additional dose of CyP, the graft stopped beating on the 13th postoperative day, in the presence of a total APAb level of 971 units. Histological examination of this graft demonstrated AVR. The elicited post-transplant total APAb level in heterotopic 2 remained low and on the second postoperative day when the baboon was sacrificed, having experienced a cerebrovascular accident, the total APAb level was 56 units. Histological examination of this graft demonstrated no evidence of rejection. The elicited post-transplant total APAb level in heterotopic 3 remained low until the 20th postoperative day when there was a small increase in the level to 122 units. At this time an additional dose of CyP was administered but by the 21st postoperative day it was clear that the baboon had developed a cervical abscess and so it was sacrificed. At the time of sacrifice the total APAb level had fallen to 60 units and histological examination of the xenograft demonstrated no evidence of rejection.

The elicited post-transplant total APAb level in orthotopic 1 remained suppressed throughout its course but the animal was sacrificed with a beating life-supporting cardiac xenograft on the ninth postoperative day due to poor general condition and having developed widespread petechial bruising. At the time of sacrifice the total APAb had risen to 177 units but histological examination of the xenograft

demonstrated no evidence of rejection. In both orthotopics 2 and 3 the elicited total APAb levels remained suppressed until the fifth postoperative day at which time the xenografts stopped beating in the presence of rising total APAb antibody levels of 104 units and 718 units respectively. Histological examination of both xenografts demonstrated AVR.

Histology

All transgenic donor hearts scored 4 (scale of 0 to 4) for hDAF expression.

The results of the grading for the histological parameters for heterotopic and orthotopic xenografts can be seen in table 4.3.

Histological examination of the first heterotopic cardiac xenograft, which stopped beating on day 13, demonstrated the typical features (although mild) of AVR i.e. rupture of the endothelial cell layer, oedema, thrombosis, haemorrhage and focal necrosis of the cardiomyocytes, with an infiltrate of both polymorphonuclear cells and monocytes.

Xenografts from heterotopic recipients 2 and 3, which were sacrificed with beating cardiac xenografts, demonstrated no evidence of rejection.

Histological examination of the first orthotopic cardiac xenograft which was sacrificed on the ninth postoperative day, due to widespread petechial haemorrhage and poor general condition, demonstrated no evidence of rejection (figure 4.4). Indeed normal myocardial architecture was maintained and there was no cellular infiltrate.

The first cardiac xenograft {orthotopic 3(1)} transplanted into the third orthotopic recipient failed after only 20 minutes without obvious cause and so a second cardiac xenograft was implanted {orthotopic 3(2)} which stopped beating after five days. Histological examination of orthotopic 3(1) demonstrated no evidence of rejection with normal myocardial architecture and no cellular infiltrate. Histological examination of orthotopic 3(2) demonstrated florid features of AVR as did orthotopic 2 i.e. rupture of

the endothelial cell layer, oedema, thrombosis, haemorrhage and myocytolysis with a predominantly lymphocytic infiltrate (figure 4.5).

Histological examination of both grafts which failed for technical reasons i.e. orthotopics 4 and 5 demonstrated no evidence of HAR with no evidence of graft thrombosis, endothelial cell rupture, endocardial damage or myocyte damage. Examination of orthotopic 4 confirmed pulmonary artery thrombosis but no other abnormality and examination of orthotopic 5 demonstrated a left ventricular infarction.

Immunohistology

The results of the grading for the immunohistological parameters and cell counts for heterotopic and orthotopic xenografts can be seen in table 4.4.

Histological examination of the first heterotopic cardiac xenograft, which underwent mild AVR on the 13th postoperative day, demonstrated heavy staining for IgG (4+). There was moderate deposition of C3 (3+) and C4 (3+) and mild deposition of C9 (2+). There was a heavy deposition of VWF (4+) and mild staining for P selectin (2+). The mean number of tissue platelets per high power field was nine and there was a heavy cellular infiltrate of neutrophils (63/HPF), CD-68 macrophages (58/HPF), CD-4 T helper cells (36/HPF) and CD-8 T cytotoxic cells (48/HPF). There was a slight infiltrate of CD-19 B cells (4/HPF).

Xenografts from heterotopic recipients 2 and 3, however, which were sacrificed with beating cardiac xenografts, demonstrated no evidence of rejection. Moderate staining for IgG (3+) was present in both. There was equivocal deposition of C3 (1+), mild C4 (2+) and no C9 in both grafts. Mild deposition of VWF (2+) and no staining for P selectin was seen in both grafts. The mean number of tissue platelets per high power field in heterotopics 2 and 3 was three and five respectively. A mild cellular infiltrate of neutrophils (10/HPF), CD-68 macrophages (18/HPF) and CD-8 cytotoxic T cells (5/HPF) was seen in heterotopic 2 which survived for only two days. In contrast there

were no CD-4 T helper cells and no CD-19 B cells in the xenograft of heterotopic 2. In heterotopic 3, which survived for 21 days there was a moderate cellular infiltration of neutrophils (25/HPF) and CD-68 macrophages (28/HPF) and a mild infiltration of CD-4 T helper cells (2/HPF). There were no CD-8 cytotoxic T cells or CD-19 B cells in this xenograft.

Histological examination of the first orthotopic cardiac xenograft, which was sacrificed on the ninth postoperative day due to widespread petechial haemorrhage and poor general condition, demonstrated no evidence of rejection. There was moderate staining for IgG (3+) and mild staining for IgM (2+). There was equivocal deposition (1+) of C3, mild C4 (2+), no C9 (figure 4.6), mild deposition of VWF (2+) and no staining for P selectin. Finally, there were no tissue platelets seen and there was no cellular infiltrate.

Xenografts from orthotopic recipients 2 and 3(2), both stopped beating on day five and histological examination demonstrated florid AVR in both. A moderate deposition of IgG (3+) and a heavy deposition of IgM (4+) was present in both grafts. A heavy (4+) deposition of C3, C4 and C9 (figure 4.6) was seen in both grafts. Both grafts also had a heavy deposition of VWF (4+) and there was mild staining for P selectin (2+) in orthotopic 2 and none in orthotopic 3(2). The mean number of tissue platelets per high power field in orthotopics 2 and 3(2) was eight and 18 respectively. There was a heavy infiltrate of CD-4 T helper cells, 14+42/HPF, CD-8 cytotoxic T cells, 10+59/HPF and CD-68 macrophages, 85+71/HPF. No CD-19 B cells were seen in either graft.

Histological examination of orthotopic 3(1) which failed after 20 minutes without obvious cause demonstrated no evidence of HAR. There was a heavy deposition of IgG (4+) and IgM (3+), a mild deposition of C3 (2+), heavy deposition of C4 (4+) and no C9. The deposition of VWF (2+) was mild and equivocal staining for P

selectin (1+) was observed. There were no tissue platelets seen in the xenograft of orthotopic 3(1).

Histological examination of both grafts which failed for technical reasons i.e. orthotopics 4 and 5 demonstrated no evidence of HAR. These grafts demonstrated a moderate deposition of IgG (3+) and heavy deposition of IgM (4+) anti-pig antibodies (figure 4.7a). Deposition of C3 (2+) was mild, C4 (4+) heavy, while that of C9 (1+) was equivocal in both (figure 4.7b). There was no staining for P selectin in either graft and a mild deposition of VWF (2+) was seen in xenografts of orthotopic 4 and 5.

No tissue platelets were seen in either graft and no cellular infiltrate of any kind was demonstrated in orthotopic 4. In orthotopic 5 there was no cellular infiltrate other than a very mild infiltrate of CD-68 macrophages (2/HPF).

Bone marrow examination

Bone marrow from the first orthotopic recipient which was sacrificed on day nine, having developed widespread petechial haemorrhages and a profound pancytopenia, demonstrated marked bone marrow suppression with severely depleted leukoerythroblastic precursors. Bone marrow from the two other orthotopic baboon recipients which survived for five days (i.e. orthotopics 2 and 3) demonstrated no evidence of marrow suppression with a normal leukoerythroblastic picture.

Recipient	Het 1	Het 2	Het 3	Orth 1	Orth 2	Orth 3(1)	Orth 3(2)	Orth 4	Orth 5
Outcome	13 d	2 d	21 d	9 d	5 d	20 m	5 d	11 h	6 h
Retracted Endothelial Cells	2	0	0	0	4	0	4	0	0
Wbc Adherence	2	0	0	0	4	0	4	0	0
Endothelial Permeability	2	0	0	0	4	0	4	0	0
Endothelial Rupture	1	0	0	0	1	0	3	0	0
Vasculitis	1	0	0	0	4	0	3	0	0
Congestion	3	2	1	1	4	0	4	0	4
Thrombosis	2	0	0	0	4	0	4	0	0
Platelet Thrombi	1	0	0	0	4	0	3	0	0
Fibrin	4	1	2	1	4	0	4	0	2
Inflammatory Cells	2	0	0	0	4	0	3	0	0
Myocyte Damage	2	0	0	0	4	0	4	0	0
Myocytolysis	0	0	0	0	4	0	4	0	0
Focal Necrosis	1	0	0	0	2	0	3	0	0
Diffuse Necrosis	0	0	0	0	0	0	3	0	0
Perivascular Infiltrate	2	0	0	0	4	0	0	0	0
Focal Infiltrate	2	0	0	0	0	0	0	0	0
Diffuse Infiltrate	0	0	0	0	0	0	3	0	0
Aggressive Infiltrate	0	0	0	0	2	0	3	0	0
Focal Haemorrhage	2	2	1	0	3	1	4	0	3
Diffuse Haemorrhage	0	2	0	0	2	0	3	0	1
Oedema	3	2	2	0	2	1	3	0	1
Endocardial Adherence	0	0	0	0	0	0	2	0	0
Endocardial Permeability	0	0	0	0	0	0	2	0	0
Endocardial Disruption	0	0	0	0	0	0	0	0	0
Hyperacute Rejection	No	No	No	No	No	No	No	No	No
Acute Vascular Rejection	Yes	No	No	No	Yes	No	Yes	No	No
No Evidence of Rejection	No	Yes	Yes	Yes	No	Yes	No	Yes	Yes

Table 4.3. Histological grading and outcome for heterotopic and orthotopic baboons. All histological parameters were graded according to intensity of staining on a scale of 0 to 4. Het=heterotopic, Orth=orthotopic, m=minutes, h=hours, d=days.

Note none of the grafts hyperacutely rejected. Orthotopic graft 1, which supported the life of a baboon for nine days, demonstrated no structural damage or cellular infiltrate. However heterotopic graft 1 and orthotopic grafts 2 and 3 (2) showed significant graft damage due to acute vascular rejection.

Recipient	Het 1	Het 2	Het 3	Orth 1	Orth 2	Orth 3(1)	Orth 3(2)	Orth 4	Orth 5
Outcome	13 d	2 d	21 d	9 d	5 d	20 m	5 d	11 h	6 h
C3	3	1	1	1	4	2	4	2	2
C4	3	2	2	2	4	4	4	4	4
C9	2	0	0	0	4	0	4	1	1
IgG	4	3	3	3	3	4	3	3	3
IgM	–	–	–	2	4	3	4	4	4
P selectin	2	0	0	0	2	–	0	0	0
Von Willebrand's Factor	4	2	2	2	4	–	–	2	–
GSL.1	4	3	3	–	–	–	–	–	–
	Mean number of cells /HPF								
Tissue Platelets	9	3	5	0	8	–	18	0	0
Tissue Neutrophils	63	10	25	–	–	–	–	–	–
CD-4 T Cells	36	0	2	0	14	–	42	0	0
CD-8 T Cells	–	5	0	0	10	–	59	0	0
CD-68 Monocytes	58	18	28	0	85	–	71	0	2
CD-19 B Cells	4	0	0	0	0	–	0	0	0
Hyperacute Rejection	No	No	No	No	No	No	No	No	No
Acute Vascular Rejection	Yes	No	No	No	Yes	No	Yes	No	No
No Evidence of Rejection	No	Yes	Yes	Yes	No	Yes	No	Yes	Yes

Table 4.4. Immunohistological grading and outcome for heterotopic and orthotopic baboons. All histological parameters were graded according to intensity of staining on a scale of 0 to 4 or counted as mean number of cells / HPF (high power field).

Het=heterotopic, Orth=orthotopic, m=minutes, h=hours, d=days.

HAR=Hyperacute rejection, AVR=Acute vascular rejection, NER=No evidence of rejection.

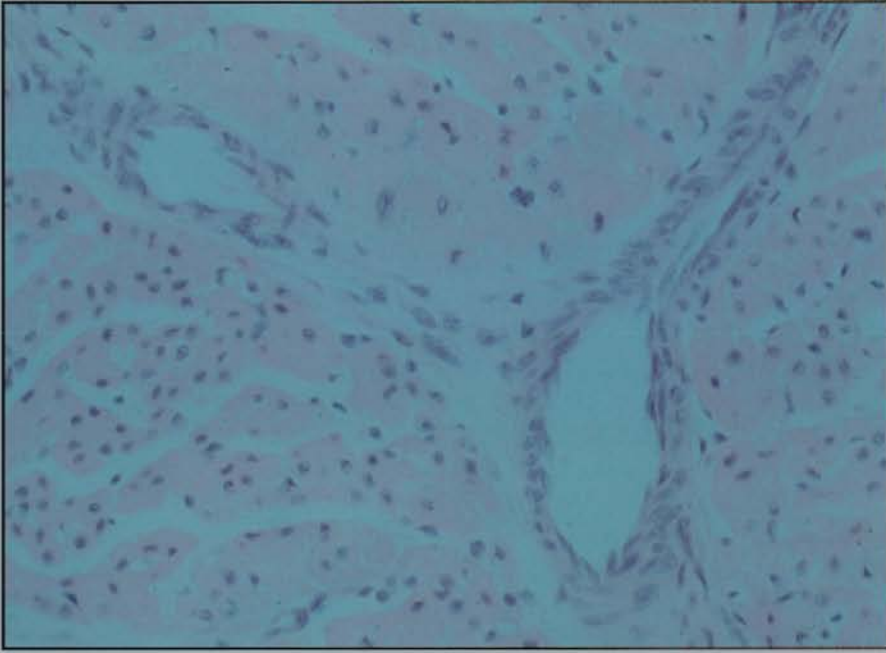


Figure 4.4. Section of cardiac tissue, stained with haematoxylin and eosin, from an hDAF transgenic pig heart transplanted orthotopically into an immunosuppressed baboon demonstrating no evidence of rejection after nine days (x20).

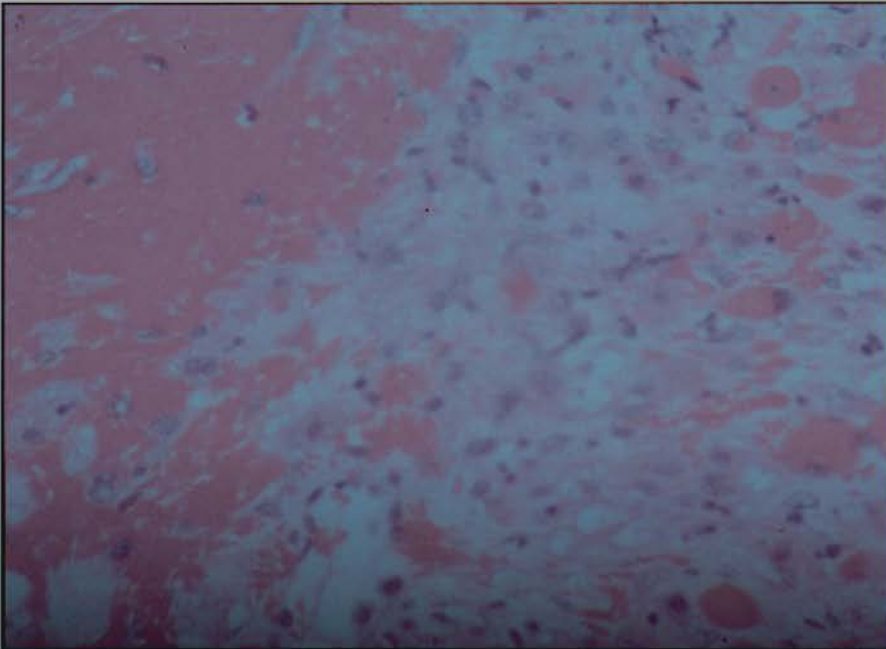
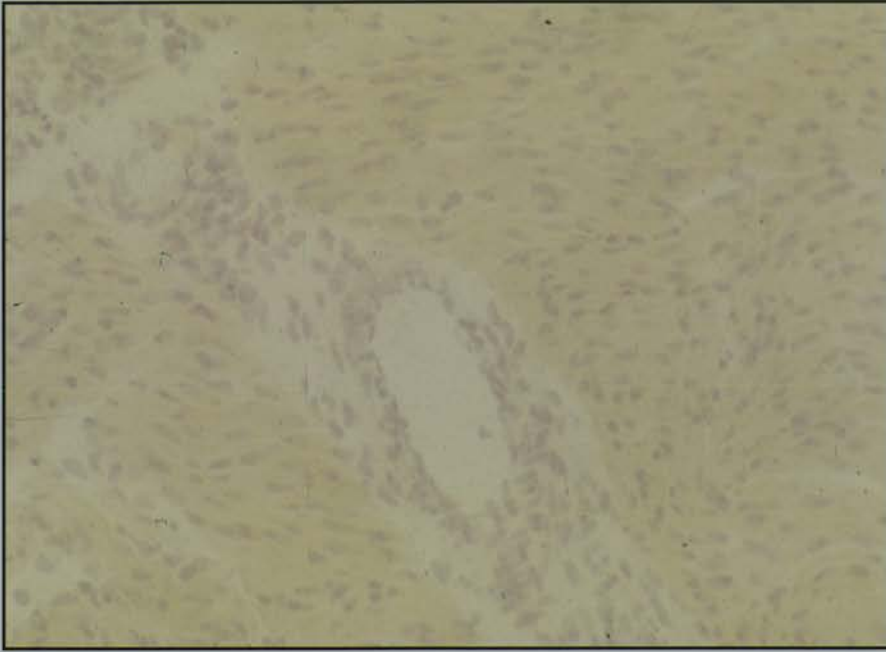
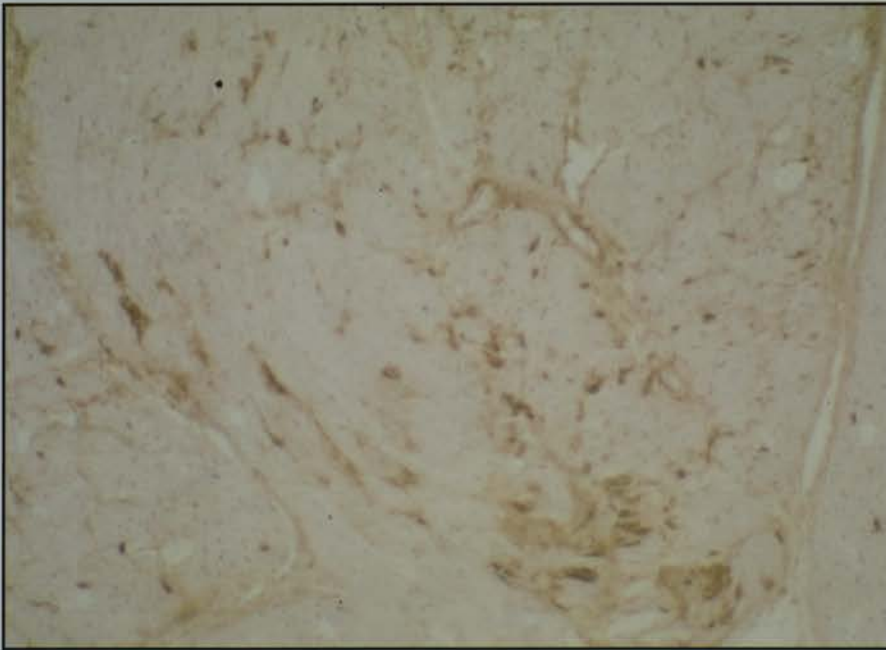


Figure 4.5. Section of cardiac tissue, stained with haematoxylin and eosin, from an hDAF transgenic pig heart transplanted orthotopically into an immunosuppressed baboon demonstrating acute vascular rejection after five days (x20).

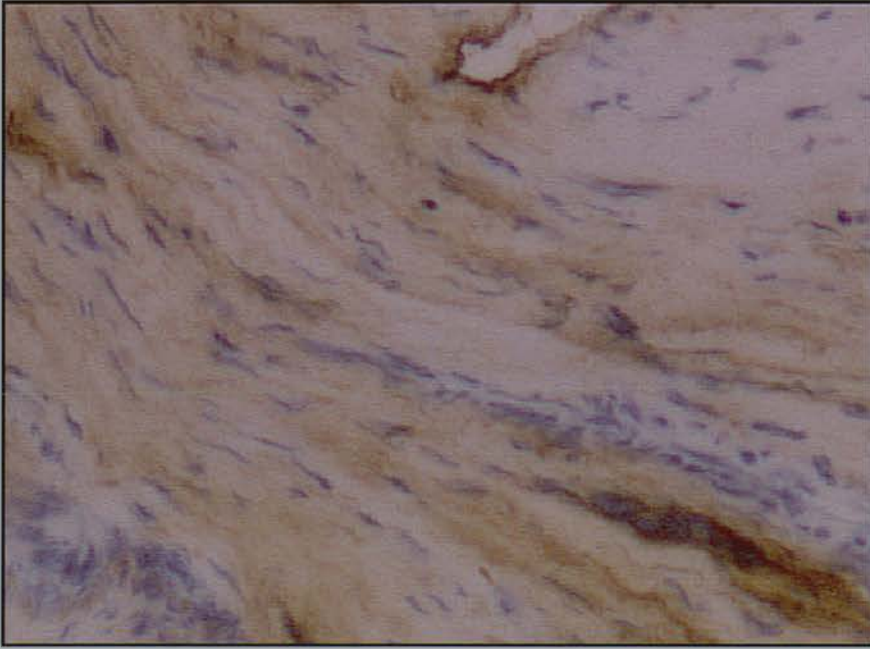


Recipient W213 surviving for nine days showing no C9 deposition (x20).

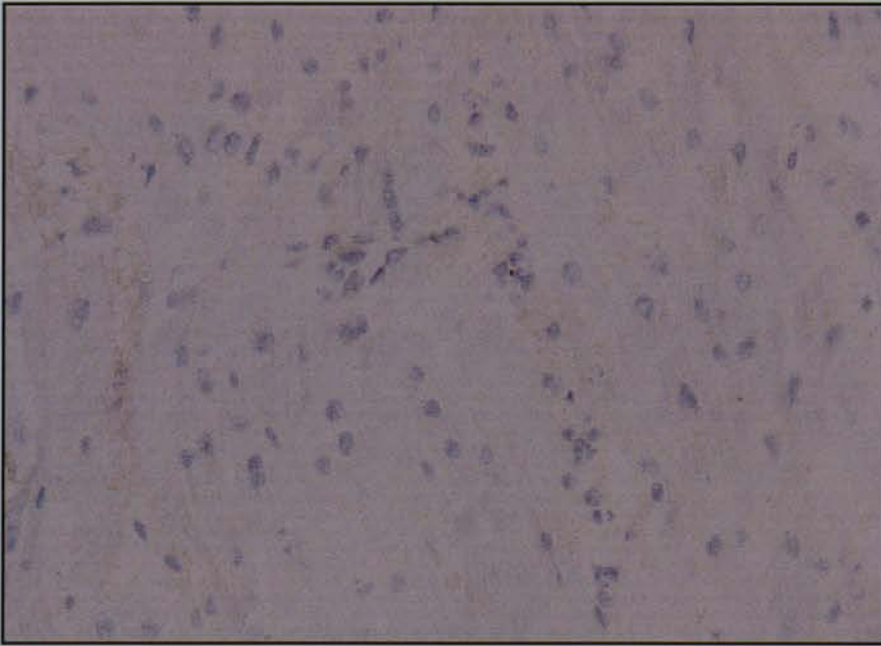


Recipient W211 surviving for five days showing heavy C9 deposition in association with acute vascular rejection (x10).

Figure 4.6. Sections of cardiac tissue, stained for complement component C9, from an hDAF transgenic pig heart transplanted orthotopically into an immunosuppressed baboon.



(a) Cardiac tissue stained for IgG (x10).



(b) Cardiac tissue stained for complement component C9 (x10).

Figure 4.7. Sections of cardiac tissue from an hDAF transgenic pig heart transplanted orthotopically into an immunosuppressed baboon to show IgG binding to graft without C9 activation.

Discussion

It is clear from these results that HAR is abrogated in pig-to-baboon cardiac xenotransplantation with the use of hDAF transgenic pigs and that hearts from these pigs are able to support the lives of primates for a prolonged period. With a combination of hDAF transgenic pig hearts and an immunosuppressive regimen of CyA, CyP and steroids the abrogation of HAR and survival of heterotopic pig-to-baboon heart xenografts for up to 21 days has been achieved. The hDAF transgenic pig heart can support the life of a primate for up to nine days when immunosuppressed with CyA, CyP and steroids and no other immune modulating strategies. Histological analysis of this graft showed normal myocardial architecture with no evidence of rejection.

Both the heterotopic and orthotopic pig-to-baboon heart transplantation models are technically very demanding and technical failures are unfortunately inevitable. Despite meticulous attention to surgical technique orthotopics 4 and 5 failed for what was thought to be technical reasons after eleven and six hours respectively. Orthotopic 3 underwent two consecutive transplants as the first xenograft 3(1) failed after only 20 minutes. No cause was found for the failure of orthotopic 3(1) and there was no evidence of rejection. The ischaemic time for this graft was, however, prolonged at 80 minutes and ischaemic injury may have resulted in early graft loss. Orthotopic 4 failed due to pulmonary artery thrombosis secondary to kinking of the pulmonary artery, as a direct result of the donor heart being too large (+57.8%). No other difference was recorded compared with those grafts which were technically successful. There was no significant difference in ischaemic time, cardiopulmonary bypass time or pretransplant total APAb level (table 4.2). In every orthotopic transplantation the donor pig heart was larger than the recipient baboon heart with a weight mismatch ranging from +5% to +57.8%, despite the body weight of the donor pig being less than that of the recipient baboon in every case. Experience obtained in this study indicated that the

heart/body weight ratio of pigs is greater than that of baboons. Therefore, to match pig and baboon heart sizes, and so make the operation technically less difficult, the body weight of donor piglets should be 20-30% less than the recipient baboon weight.

Orthotopic 5 died of graft failure due to dysrhythmia following left ventricular infarction and subsequent systemic acidosis six hours following surgery. The limited postoperative care of the recipients without electrocardiogram, blood pressure and central venous pressure monitoring made it difficult to determine the exact cause of the early graft failure. It should be noted that there was a +30.6% weight mismatch in this recipient and no difference in the cardiopulmonary bypass time, ischaemic time or pretransplant total APAb compared with those technically successful orthotopic grafts.

In chapter three we found that although the high CyP regimen received by the group 1 cynomolgus recipients resulted in more side effects, the xenograft survival was significantly greater than in the low CyP, group 2, recipients all of which underwent AVR. The group 1 CyP regimen was therefore chosen for the immunosuppression of the baboons. CyP was given intravenously on the preoperative day at 40mg/kg, the operative day at 20mg/kg, the second postoperative day at 10-40mg/kg and alternate days thereafter, tailored to the white count and the total haemolytic red blood cell APAb levels. CyP was administered to achieve a minimum total wcc of $2 \times 10^6/\text{ml}$. This minimum wcc reflects experience which has recently been gained in clinical immunosuppression of patients suffering from autoimmune diseases such as systemic lupus erythematosus.⁸⁷ Some authors even recommend a wcc as low as $1.5 \times 10^6/\text{ml}$ as the minimum acceptable level for immunosuppression in patients with lupus nephritis.¹²² The first orthotopic baboon, however, developed bone marrow suppression and so the CyP dose in the other two successful orthotopic baboons was reduced, each baboon receiving a total of 10mg/kg. The mean daily dose of CyP received by all baboons was $11.1 \pm 2 \text{mg/kg}$. This compared with a mean daily dose of $17.9 \pm 3.6 \text{mg/kg}$ in group 1 cynomolgus monkeys in chapter three. Clearly baboons

either absorb CyP better than cynomolgus monkeys or their bone marrow leukoerythroblastic precursors are more sensitive to it. It is noteworthy that the baboons, unlike the group 1 cynomolgus monkeys, did not develop diarrhoea and this might explain the smaller doses of CyP required to achieve the set criteria. It would, however, still appear that baboon bone marrow is more sensitive to CyP than cynomolgus monkey bone marrow as the total wcc in the baboons had fallen by the fourth postoperative day to below the set threshold level of $2 \times 10^6/\text{ml}$ and failed to recover despite the reduction in CyP dose (figure 4.1). In cynomolgus monkeys by careful manipulation of the CyP dose we were able to maintain the total wcc at about the threshold level. A similar pattern was also seen with the lymphocyte count in baboons.

Data exists to suggest that CyA trough levels in baboons should be as high as 1500ng/ml ¹²¹ and in these baboon models we aimed for trough levels in excess of 600ng/ml . The actual mean CyA level achieved in the baboons was $987 \pm 76\text{ng/ml}$. This required a mean daily dose of $177 \pm 12.7\text{mg/kg}$. This compared with group 1 cynomolgus monkeys in chapter three in which we achieved a mean CyA level of $544 \pm 71\text{ng/ml}$ with a mean daily dose of $122 \pm 19\text{mg/kg}$. This represented similar CyA dosing in both cynomolgus monkey and baboon to achieve a given CyA trough level.

The pretransplant APAb levels in the baboons ranged from 430 units to 1152 units and in each case fell almost to zero immediately following transplantation, presumably due to immunoabsorption onto the graft. It should be noted that in orthotopic 3 the pre transplant total APAb level started at 1152 units and that the first pig heart grafted failed with no obvious cause identified. During the same anaesthetic a second pig heart was transplanted successfully which went on to support the life of the recipient baboon for five days. Following the explant of the first transplanted heart and prior to implantation of the second heart the total APAb level was found to be 98 units and as such the second graft may have been relatively protected against anti-pig antibodies by

the initial perfusion of baboon blood through the first pig heart thus facilitating immunoabsorption of anti-pig antibodies onto the graft.

There was a large variation in the elicited APAb level from one baboon to the next. Not only did the elicited APAb level vary widely but the effect of a given antibody level on the graft also varied. However, in every case where the graft was lost due to rejection it was preceded by a rise in total APAb level to a variable degree. Heterotopic 1 was rejected on the 13th postoperative day in the presence of a total APAb level of 971 units, while orthotopic 2 was rejected in the presence of a total APAb level of only 104 units (risen from 9 units the previous day). This would suggest that in this model the development of AVR is an antibody mediated phenomenon and it is likely that there is more than one antibody, with different affinities, involved. We have already shown in chapter two that in the non-immunosuppressed cynomolgus recipient of a pig heart delayed xenograft rejection or AVR was preceded by a rise in total APAb level which was mirrored by a rising anti-gal α 1-3 gal IgM level (see chapter two).

Histological examination of the first orthotopic xenograft which was sacrificed with a beating life supporting cardiac xenograft on the ninth postoperative day demonstrated no evidence of rejection and indeed normal myocardial architecture was maintained and there was no cellular infiltrate. However, histological examination of bone marrow from this animal demonstrated profound marrow suppression with grossly depleted leukoerythroblastic precursors.

Those heterotopic and orthotopic grafts which were rejected demonstrated a histological pattern first described in a discordant rodent model of complement-depleted recipients and referred to as either AVR,⁸¹ or delayed xenograft rejection.¹²³ A cellular infiltrate consisting predominantly of macrophages (CD-68) and neutrophils was found. CD-4 T-helper and CD-8 T-cytotoxic cells were also present in smaller numbers.

Marked C4 deposition was seen in all grafts but C9 was only detected (other than 1+) in those three grafts which were rejected which suggests that terminal complement pathway activation contributed to graft rejection. It is possible that insufficient immunosuppression allowed the formation of high affinity induced anti-xenograft antibodies which overwhelmed the complement inhibiting properties of hDAF. Although the measurement of haemolytic anti-pig antibodies in the serum does not accurately reflect what is happening at the level of the xenograft, the antibody peaks that were observed in these animals at the time of rejection would support this. It would appear that activation of terminal complement components via the classical pathway plays an important role in the pathogenesis of AVR and that hDAF has a limited ability in preventing this.

In those grafts which were rejected there was clear evidence of activation of the clotting cascade with VWF deposition. In all grafts there was little P selectin staining, reaching only as high as grade 2+ in those grafts which were rejected. This confirms our findings in chapter two which suggested that hDAF, in addition to protecting against HAR, also suppresses endothelial cell activation.

Histological examination of those grafts which failed for technical reasons at six and eleven hours following surgery, interestingly, showed strong IgG and IgM binding to the grafts, moderate C3, very heavy deposition of C4 and only slight deposition of C9 confirming that there was substantial antibody binding to the graft but that this did not result in the activation of the terminal component of the complement cascade, as would be expected from the point of action of DAF (see chapter 1). It is clear from these results that HAR is abrogated in pig-to-baboon cardiac xenotransplantation with the use of hDAF transgenic pigs and that hearts from these pigs are able to support the lives of primates for a prolonged period. We have, however, also seen that CyP when administered at a mean daily dose of 15mg/kg was associated with toxicity and bone marrow suppression and that if this mean daily dose was reduced to 10mg/kg an

elicited APAb response occurred around day five which resulted in loss of the graft due to AVR. The large doses of CyA and CyP administered in these studies, compared with doses employed clinically, were necessary to produce the desired serum CyA levels and suppression of recipient white cells. Such dosing was thought to be necessary due to the relatively poor absorption of these agents in non-human primates. We anticipate that with the advent of new immunosuppressive agents specifically targeting this antibody response, improved survival of hDAF transgenic pig organs in primates will be achieved and that clinical xenotransplantation with hDAF transgenic pig organs will be possible.

Chapter Five

Discussion and Conclusions

Heterotopic abdominal heart transplantation from neonatal pig-to-cynomolgus monkey, heterotopic cervical heart transplantation from neonatal pig-to-baboon and orthotopic heart transplantation from neonatal pig-to-baboon are exacting surgical techniques associated with a significant technical failure rate. Despite this, we have demonstrated that none of the hDAF transgenic grafts were hyperacutely rejected and clearly hDAF protects pig cardiac xenografts from hyperacute rejection in cynomolgus monkey and baboon. However, hyperacute rejection in the discordant pig-to-cynomolgus monkey xenotransplantation model is not inevitable. Indeed only half of the non-immunosuppressed control grafts were hyperacutely rejected with a median beating time of 58 minutes and half underwent acute vascular rejection with a median beating time of 3.25 days. Those non-immunosuppressed control hearts which were not hyperacutely rejected were found to have significantly less GSL.1 binding, and hence glycosylated sugars, on the endothelium of their grafts. It is this finding i.e. the relative lack of antigenicity in this group, which may help to explain why these control animals survived far in excess of what was expected.

All cynomolgus monkey and baboon recipients had a high preformed natural anti-pig antibody (APAb) level prior to transplantation which fell almost to zero immediately following transplantation and which we believe was due to immunoabsorption onto the graft. In support of this conclusion, there was a substantial amount of IgG bound to those transgenic and control technical failure grafts (failing within a few hours of transplantation) in both the cynomolgus monkeys and baboons and a substantial amount of IgM bound to the technical failure grafts in the baboons. The fall in the total APAb level immediately following transplantation was mirrored by a fall in anti-galactose α 1-3 galactose (AAG) IgM but not IgG antibodies in the non-immunosuppressed cynomolgus monkey and therefore it would appear that it was the immunoabsorption of AAG IgM onto the graft which was the trigger for complement activation and hyperacute rejection.

The elicited total APAb level in the non-immunosuppressed cynomolgus monkeys remained low until about the third postoperative day when it started to rise rapidly resulting in graft loss in all cases by the fifth postoperative day. Graft loss in all cases was due to acute vascular rejection (AVR). The hDAF transgenic grafts were able to tolerate higher levels of elicited total APAb compared with the long-surviving controls before succumbing to AVR. Clearly, hDAF conferred on the graft some degree of protection against antibody mediated AVR. In the group 1, high dose cyclophosphamide, immunosuppressed cynomolgus monkeys the elicited total APAb levels remained low until about the third postoperative day, at which time they began to rise and then tended to peak by the fifth postoperative day. Beyond the fifth postoperative day there were a variable number of peaks and troughs of antibody levels. The amplitude of these peaks also varied widely. Despite the many antibody peaks, however, only two resulted in xenograft rejection. In contrast, the pattern of elicited total APAb levels and their effect on graft viability in the group 2, low dose cyclophosphamide, cynomolgus monkeys was markedly different. The levels initially remained low until about the fifth to eighth postoperative days, at which time there was again an increase in antibody levels in four out of five recipients, resulting in every case in AVR. There was only one recipient in group 2 in which there wasn't an antibody increase around this time, but a later peak in antibody level resulted in AVR of the graft. It would appear that perioperative cyclophosphamide in some way protected the grafts from later antibody attack, or that there may have been more than one type of APAb involved such that recipients in group 1 were able to tolerate such peaks of antibody whereas group 2 recipients were not.

In the baboon models there was a large variation in the elicited APAb level from one baboon to the next. Not only did the elicited APAb level vary widely but the effect of a given antibody level on the graft also varied. However, in every case where the graft was lost due to rejection it was preceded by a rise in total APAb level to a variable

degree. These results would suggest that the development of AVR is, at least in part, an antibody mediated phenomenon and it is likely that there is more than one antibody, with different affinities, involved.

In non-immunosuppressed cynomolgus recipients of pig hearts AVR was preceded by a rise in total APAb level which was mirrored by a rising AAG IgM level and not by the AAG IgG level. This suggests that, at least in the non-immunosuppressed cynomolgus monkey, it was AAG IgM which was in part responsible for the development of AVR.

We have shown that prolonged survival of heterotopic hDAF transgenic pig hearts can be achieved both in the pig-to-cynomolgus monkey and in the pig-to-baboon models with a simple immunosuppressive regimen of cyclophosphamide, cyclosporin A and steroids. However, it would appear that cynomolgus monkeys do not absorb cyclosporin A and cyclophosphamide as well as humans and so the doses of these drugs had to be increased to levels which, by clinical standards, were high, but which resulted in satisfactory cyclosporin A levels and haematological responses similar to those seen in clinical practise. Such large doses of immunosuppressive agents were not without their side effects. Indeed, 40% of cynomolgus recipients treated with the group 1, high dose cyclophosphamide, regimen were sacrificed because of the development of intractable diarrhoea, thought to be due to cyclophosphamide toxicity. The omission of perioperative cyclophosphamide in the group 2, low dose cyclophosphamide, immunosuppressed cynomolgus monkeys resulted in a 44% reduction in the amount of cyclophosphamide administered, and although none of the monkeys developed diarrhoea, all cardiac xenografts were lost due to AVR and so it would appear that perioperative cyclophosphamide was an essential component of this regimen. In the baboon model it would appear that baboons either absorb cyclophosphamide better than cynomolgus monkeys or baboon bone marrow is more

sensitive to it as smaller doses of cyclophosphamide were needed in baboons than in cynomolgus monkeys to achieve the same degree of immunosuppression.

We have also shown that the hDAF transgenic pig heart is able to support the life of a baboon, immunosuppressed with cyclosporin A, cyclophosphamide and steroids, for a prolonged period.

Histological analysis of all xenografts has demonstrated that hDAF protects transgenic pig hearts from damage from terminal components of the complement cascade. Immunohistological analysis of all control hearts, compared with transgenic hearts, demonstrated no difference in the amount of endothelial C4 deposition but significantly more endothelial C3 and C9 deposition in the control grafts. This pattern is exactly as would be predicted from the mode of action of DAF since this complement regulator acts at the point of formation of the C3 convertases C4b2a and C3bBb. Histological examination of those hDAF transgenic grafts which were lost due to AVR in both the cynomolgus monkey and baboon models demonstrated heavy deposition of C3 and C9 in the grafts supporting the hypothesis that complement activation is not only responsible for hyperacute rejection but also plays an important part in the pathogenesis of AVR.

It would also appear that hDAF protects transgenic pig hearts from endothelial cell activation as indicated by significantly less staining for the adhesion molecule P selectin in the grafts of immunosuppressed cynomolgus monkeys and in the technical failure transgenic grafts of non-immunosuppressed cynomolgus monkeys. And, indeed, hDAF also protects transgenic pig hearts from damage from activation of the coagulation cascade as indicated by significantly less fibrin deposition in the transgenic technical failure grafts of non-immunosuppressed cynomolgus monkeys and significantly less Von Willebrand's Factor deposition in transgenic grafts of immunosuppressed cynomolgus monkeys.

In all models described, those cardiac xenografts which were not either hyperacutely rejected or whose recipients were not sacrificed for humane reasons were lost due to AVR and in every case there was a cellular infiltrate consisting predominantly of macrophages (CD-68) and neutrophils. CD-4 T-helper and CD-8 T-cytotoxic lymphocytes were also present in smaller numbers. With appropriate immunosuppression this infiltrate could be prevented in both the cynomolgus and baboon models.

Finally, it is clear from these results that

- a) hyperacute rejection can be abrogated in the pig-to-cynomolgus monkey and pig-to-baboon models with the use of hearts transgenic for hDAF
- b) prolonged survival can be achieved in these models with appropriate immunosuppression and
- c) hDAF transgenic pig hearts can support the life of a non-human primate for a prolonged period.

However, it is also clear that antibody mediated acute vascular rejection poses the next major hurdle to pig-to-primate xenotransplantation.

Future developments

The combination of further human regulators of complement activation with hDAF, for example CD59, might be able to prevent antibody mediated acute vascular rejection. Already human CD59 and hDAF transgenic pigs have been developed.

The additional use of immunoabsorbent columns for the removal of both preformed naturally occurring and elicited anti-pig antibodies may also be useful in preventing antibody mediated xenograft rejection. In particular, immunoabsorbent columns for the removal of galactose α 1-3 galactose IgG and IgM are likely to be effective.

In vivo infusion of soluble galactose α 1-3 galactose may also be of help in overcoming the fierce anti-gal antibody response to the xenograft by neutralising circulating anti-xenograft antibodies.

And finally, we anticipate that with the advent of new immunosuppressive agents (e.g. leflunomide, brequinar, rapamycin, deoxyspergualin and mycophenolate mofetil) specifically targeting this antibody response, improved survival of hDAF transgenic pig organs in primates will be achieved and that clinical xenotransplantation with hDAF transgenic pig organs might be possible.

Since the experimental work presented in this thesis was completed our knowledge of xenotransplantation has grown .

Numerous approaches have been reported for removing preformed natural antibodies.¹²⁴ However, all such approaches result only in a transient fall in antibody level and a later rebound to levels above the baseline. Therefore more recent efforts to deplete the recipient's natural antibodies have begun to concentrate on attempts to deplete the B-1 population of B cells entirely or to induce tolerance in this population for the gal α 1-3 gal determinant.¹²⁵

Since expression of the gal α 1-3 gal determinant depends on the expression of the galactosyl transferase enzyme, one approach to removing this determinant has been to eliminate the galactosyl transferase gene by homologous recombination. Knock-out mice lacking this gene have been produced.¹²⁶ The technology for this approach is not yet available in other species.

Cellular immune responses to xenografts are considerably stronger than comparable responses to allografts. It is unlikely that currently available immunosuppressive agents will either be potent enough or specific enough to suppress the xenogeneic response in discordant xenotransplantation. For this reason, the success of clinical xenotransplantation is likely to depend, at least in part, on finding ways of inducing specific hyporesponsiveness, or tolerance, across xenogeneic barriers rather than

relying entirely on non-specific immunosuppressive agents. However, the only reported approach toward induction of specific tolerance for the clinically relevant pig-to-primate species combination involves attempts to induce mixed chimerism through bone marrow transplantation.¹²⁷

These results suggest that combined approaches to control both the humoral and cellular xenograft responses may eventually permit long-term acceptance of discordant xenografts in the pig-to-primate combination, making clinical xenotransplantation a reality.

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